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MORRIS M. ATTA
Deputy to the Chairperson

State of Hawaii
DEPARTMENT OF AGRICULTURE
1428 South King Street
Honolulu, Hawaii 96814-2512
Phone: (808) 973-9600 FAX: (808) 973-9613

December 10, 2021

TO: Advisory Committee on Plants and Animals

FROM: Dr. Martin Nadeau, Scientific Director
Anatis Bioprotection Inc.

THROUGH: Wil Leon Guerrero
Microorganism Specialist
Plant Quarantine Branch
Hawaii Department of Agriculture

Subject: Request to: (1) Preliminary Review for a Change in the List Placement of *Beauveria bassiana* strain ANT-03, a fungi on the List of Restricted Microorganisms (Part A), for Future Placement on the List of Nonrestricted Microorganisms, for use in a Microbial Bio-Remediation Product.

I. Summary Description of the Request

PQB NOTES: *The Plant Quarantine Branch (PQB) submittal for requests for import or possession permits, as revised, distinguishes information provided by the applicant from procedural information and advisory comment and evaluation presented by PQB. With the exception of PQB notes, hereafter "PQB NOTES," the text shown below in Section II from page 6 through page 7 of the submittal was taken directly from Anatis Bioprotection's application and subsequent written communications provided by the applicant, Dr. Martin Nadeau. For instance, the statements on page 6 regarding effects on the environment are the applicant's statements in response to standard PQB questions and are not PQB's statements. This approach for PQB submittals aims for greater applicant participation in presenting import requests in order to move these requests to the Board of Agriculture (Board) more quickly, while distinguishing applicant provided information from PQB information. The portion of the submittal prepared by PQB, including the Summary of Proposed Rule Amendments and Advisory Subcommittee Review is identified as Sections III and IV of the submittal, which start at the beginning of page 12 and end of page 12.*

We have a request to review the following:

COMMODITY: Bioceres WP, a wettable powder formulation of bioinsecticide containing 10 % (w/w) of conidia of *Beauveria bassiana* strain ANT-03 (equivalent to 1 x10^{10} conidia/g)

SHIPPER: Leader Liquids LLC (The manufacturer of the BioCeres WP), 345 Vista Drive, Cold Water, MI 49036

IMPORTER: Nutrien Ag Solutions, 900 Leilani Street, Hilo, HI 96720

CATEGORY: All species in the genus *Beauveria*, are on the List of Restricted Microorganisms (Part A). This includes *B. bassiana* strain ANT-03. Pursuant to chapter 4-71A, Hawaii Administrative Rules (HAR), microorganism species on the List of Restricted Microorganisms (Part A) are categorized as high-risk microorganisms, which may be allowed import into the State under a permit approved by the Board.

PQB NOTES: *Microorganisms on the List of Non-Restricted Microorganisms (NR List) are classified as low risk. HAR 4-71A-2 defines a microbial product as ". . . a manufactured product containing known cultures of microorganisms for the purpose of bioremediation or bioaugmentation, including a product such as a microbial pesticide." Pursuant to HAR 4-71A-29, "A microbial product containing only microorganisms on the list of nonrestricted microorganisms . . . may only be imported into the State through a registration with the branch." Should B. bassiana strain ANT-03 be approved for placement on the NR List, Bioceres WP would be eligible for registration and subsequent importation as a Microbial Product.*

II. Information Provided by the Applicant in Support of the Application

PROJECT: Sale of the bioinsecticide, Bioceres WP, in Hawaii

PURPOSE: Biological control of insect pests in agriculture

OBJECTIVE: The objective is to provide to the crop producer a cost-effective and safe alternative insecticidal solution for the protection of agricultural crop commodity yield and quality by controlling insect pests. Other benefits include the reduction of conventional chemical insecticides by using Bioceres WP, an organic biochemical product, that is safe for humans and the environment (including nontarget pollinators). For the user, the product also offers lack of pest resistance, is

compatible with other biocontrol agents, is not phytotoxic, and does not require a pre-harvest interval.

PROCEDURE: Bioceres WP, containing the microorganism, *Beauveria bassiana* strain ANT-03, must be diluted in water and applied to the crop by using conventional spraying equipment to cover the leaves of the plant. The detailed application procedure is described on the end-use product label (see Attachment 1). The product label includes all safety measures and precautions to follow when handling the product.

REGULATORY BACKGROUND: *Beauveria bassiana* is ubiquitous and found all over the world. Hawaii has several strains of *B. bassiana*. In December of 2010, researchers from the US Pacific Basin Agricultural Research Center, United States Department of Agriculture-Agricultural Research Service published findings of 14 isolates of *B. bassiana* collected from insects on Hawaii island alone.

Currently, there are six strains of *Beauveria bassiana* that are registered with the Environmental Protection Agency (EPA) as follows.

Active Ingredient Name	PC Code	Year First Registered
B. bassiana GHA	128924	1995
B. bassiana ATCC 74040	128818	1995
B. bassiana strain 447	128815	2002
B. bassiana HF23	090305	2006
B. bassiana strain ANT-03	129990	2014
B. bassiana strain PPRI 5339	128813	2018

Of the six strains, HF23 and ANT-03 are approved for organic use.

All *B. bassiana* strains in Agricultural Pesticide products designed for outdoor use, have similar language on the labels under the heading: ENVIRONMENTAL HAZARDS, declaring that the product is potentially harmful to honeybees and to not apply while honeybees and other insects are actively pollinating or foraging. Also, similar language that drift and runoff may be hazardous to aquatic organisms and to not apply within 50 feet of aquatic habitats such as, but not limited to, lakes, reservoirs, rivers, streams, marshes, ponds, estuaries, and commercial fishponds.

Do not apply directly to water or areas where surface water is present or to intertidal areas below the mean high-water mark. Do not contaminate water when disposing of equipment washwater or rinsate.

The EPA requires detailed data and analysis on exposure to non-target species and sites to ensure that the product is safe to use when following the pesticide label's use instructions prior to registering a product. All *B. bassiana* strains registered with the EPA are assigned the "CAUTION" signal word in the precautionary statement of the pesticide label, which means that acute oral and dermal exposure is low risk. The other signal words with higher risk of exposure are: "WARNING" and "DANGER." If a product is found to be poison, then the word "POISON" along with a Skull and Crossbones must be displayed near the signal word "DANGER."

When *B. bassiana* is applied in the field, it is sensitive to light and, therefore, breaks down quickly. The potential for leaching through the soil is very low. Several applications can be made at instructed intervals until pests are not visible. There is no limit on the number of applications or total amount used on a site provided that the label directions are followed. *B. bassiana* is not phytotoxic. The pre-harvest Interval is zero days.

Currently, there are two products containing *Beauveria bassiana* GHA strain that are registered as Microbial Products in Hawaii (registered with the Plant Quarantine Branch and Pesticide Branch), and the general public is able to purchase these products in Hawaii.

DISCUSSION:

1. Person Responsible:

The responsible person is Martin Nadeau, Ph.D. Dr. Nadeau is the Scientific Director at Anatis Bioprotection at the division of Bioinsecticide[,] since June 2016. Anatis Bioprotection is the developer and the registrant of the Bioceres WP product. Dr. Nadeau's responsibilities at Anatis Bioprotection include work on the development of formulations of bioinsecticides based on the *B. bassiana* strain ANT-03 active ingredient and obtaining registrations [from the] Pest Management Regulatory Agency (PMRA) in Canada, [and the] EPA in USA[,] including California). The master and doctorate subjects were the *evaluation of*

entomopathogenic fungi against dipteran insects, and the *physiological ecology of entomopathogenic fungi,* respectively. Copies of Dr. Nadeau's diploma, CV and letter of employment are provided in Attachment 2 (documents 507, 508, 509, and 510).

2. Manufacturing Process:

The Bioceres WP end-use product is formulated and packaged at the following facility:

Leader Liquid Packaging
345 Vista Drive
Coldwater, MI 49036
EPA Establishment No. 92957-MI-001

The manufacturing process for producing *Beauveria bassiana* strain ANT-03 technical and the end-use product, Bioceres WP, ensures consistent quantity and quality of production. Anatis qualifies each batch to ensure the potency conforms to the specification of a minimum of 1 x 10^{10} viable conidia/gram of product.

Information pertaining to the manufacturing process for both the technical material and end-use product is confidential business information and can be made available upon request.

3. Method of Disposition

To avoid waste, all material in the product container should be used by the applicator. The product will be handled in heat[-] sealable pouches with the inert ingredients. If waste[ ] cannot be avoided, dispose of remaining product in a waste disposal facility or pesticide disposal program (often such programs are run by state or local governments or by industry) (see product label in Attachment 1).

4. Abstract of Organism

Beauveria bassiana (Balsamo) Vuillemin (Division: Ascomycotina, Order: Hypocreales, Family: Cordycipitaceae) is a soil entomopathogenic fungi found around the world except Antarctica. Conidia can be found naturally on insects, leaves, in the air or in the soil. Strain ANT-03 has been isolated from a dead *Lygus lineolaris* (Tarnished plant bug) (Sherrington, QC, Canada). Copies of the strain were stored in the Canadian National Mycological Herbarium (DAOM)

hosted by the AAFC and in the American ARS Culture and Patent Culture Collection (NRRL number 50797). The Anatis Bioprotection collection stock cultures of ANT-03 strain are stored in 70% glycerol in micro-tubes at -70°C.

B. bassiana forms white/yellowish colonies having a cotton texture. The conidiogenous cells are clustered, colorless, with a globose or flask-like base and denticulate apical extension called rachis bearing one conidium per denticule, resulting in a zig-zag extension. The conidium (or conidiospore) is one-cell, hyaline, round to ovoid. Spherical shape with a diameter of 1 to 4 µm or oval shape with 1.5 to 5.5 x 1-3 µm in length. In an aerobic environment, the fungus produces conidiospores. In an anaerobic environment, the fungus produces blastospores having oval shape with 2-3 µm x 7 µm in length. Blastospores are as [ ] infectious as conidia[,] causing white muscardine.

The fungus is a parasitoid for insects. It penetrates their cuticle because of the protein expression *Chitine Binding Domain* that allows the fungus to attach on an insect's exoskeleton. Then, its production of the enzyme Pr1 allows penetration of cuticles. It grows inside their host[,] feeding on their interior[,] [ ] as nutrients, ultimately killing their host. During the process, toxins may be produced. In the case of strain ANT-03, beauvericin is not, [sic] as suggested by analysis of conidia produced in laboratory conditions (Attachment 3; document 613). The optimum of growth of the strain ANT-03 is 27°C. At the temperature higher than 35°C, the fungus will not survive. The conidia are also sensitive to the UV. Insects having a high metabolism or performing activities generating heat are not devastated by this fungus.

Beauveria bassiana can infect various orders of insect: Coleoptera, Diptera, Hemiptera, Homoptera, Lepidoptera, Orthoptera, Thysanoptera. Although the fungus is an entomopatogen generalist, each strain may be specie's [sic] specific. For instance, one can be an efficient insecticide for *Leptinotarsa decemlineata* but having a minimal impact on others such as *Coleomegilla maculate* (Attachments 4 and 5; documents 609 and 614).

Beauveria bassiana strain ANT-03 is produced (technical grade) by conventional, liquid fermentation following a solid-state fermentation on cereal grains. The harvested conidia are air-dried at room temperature. The technical grade has been registered in Canada (PMRA) and in the United States (EPA)[,] including California.

The accompanying product, Bioceres WP, is a wettable formulation registered in Canada (PMRA, Registration No. 31231) and in the United States (EPA,

Registration No. 89600-2)[,] including California. Its formulation contains only organic excipients. Organic certification has been obtained (OMRI in U.S.A. and Ecocert in Canada).

5. Effects on the Environment

Although Bioceres WP may be washed off leaves and vegetables by rain and irrigation systems, or may be sprayed deliberately on soil, in the case of uses targeting insects in soil such as white grubs, it can be anticipated that the fungus will manifest its natural behavior of low mobility (refer to Attachments 6 and 7; documents 636, 638). The likelihood for these conidia to be carried toward streams or other water bodies by irrigation or rain events is reduced by the fact that they are hydrophobic (refer to Attachment 8; document 639). This characteristic makes them buoyant, increasing their exposure to UV radiation, which is lethal to the micro-organism (refer to Attachments 9, 10, and 11; documents 605, 616, 617). In addition, the viability of conidia declines exponentially in fresh water such that viability is reduced to 30% remains at day 7 (refer to Attachment 12; document 640). When all these elements are considered together, the residual viable concentration of the micro-organism that may inadvertently get to fresh water would be so low that risk of germination would be minimal (refer to Attachment 13; document 608).

6. Non-target Organisms Testing

Guideline studies and waiver rationales were submitted to U.S. EPA and Canada PMRA to meet data requirements for non-target organisms for *Beauveria bassiana* strain ANT-03. Both EPA and PMRA determined that these data and waiver rationales were sufficient to fulfill the relevant microbial pesticide data requirements and for risk assessment purposes. Further testing of non-target organisms at higher tier levels (i.e., Tiers II, III, and IV) was not required.

Studies were conducted to determine the effects of the technical grade active ingredient, *Beauveria bassiana* strain ANT-03, on birds and terrestrial and aquatic arthropods. These studies showed that the technical grade active ingredient was not pathogenic to birds; however, it may be pathogenic/toxic to insects and daphnids. As a result, the end-use product labels contain statements identifying the potential for harm to beneficial insects and bees and that applications [should] avoid direct contact to foraging bees. The end-use product labels also contain standard statements restricting users from activities that may release *B. bassiana* strain ANT-03 into aquatic environments from its use in the greenhouse.

A brief summary of the non-target organisms studies that have been submitted to and reviewed by EPA and PMRA in support of the current registrations of *Beauveria bassiana* strain ANT-03 are provided below.

a. Birds and Mammals

Acute Oral Toxicity/Pathogenicity Study in Bobwhite Quail

This study was conducted to assess the acute oral toxicity/pathogenicity potential of *Beauveria bassiana* strain ANT-03 when administered by oral gavage at 5 mL/kg once daily for five consecutive days to bobwhite quail (refer to Attachment 14; document 501).

EPA Summary from Biopesticides Registration Action Document (*BRAD) (EPA, 2015)[1] (Attachment 15)*

The guideline study submitted for the avian oral toxicity/pathogenicity testing requirement showed no adverse effects in birds tested (Northern bobwhite, Colinus virginianus). However, the study is classified as supplemental because the birds were not tested at the maximum hazard dose. Nonetheless, *Beauveria bassiana* strain ANT-03 is not likely to grow at a bird's high body temperatures (McNab, 1966) and adverse effects to birds exposed via the oral route are considered to be unlikely.

Based on expected lack of adverse effects observed in birds exposed to *Beauveria bassiana* strain ANT-03, risk to non-target birds is not likely significant.

Acute Oral Toxicity/Pathogenicity Study in Rats

An acute oral toxicity/pathogenicity study with laboratory rats indicated no adverse effects of *Beauveria bassiana* strain ANT-03 when administered at a dose of 1.3 X 10^9 CFU/animal. An acute pulmonary toxicity/pathogenicity study was also submitted that indicated *Beauveria bassiana* strain ANT-03 was not toxic, infective, or pathogenic to rats at a dose of 1.1 x 10^9 CFU/animal. An acute intraperitoneal (IP) injection toxicity and pathogenicity study does not appear to show toxicity when administered in a single dose to rats by IP injection at 1.4 x 10^9 CFU/animal.

Some wild mammals may be exposed to *Beauveria bassiana* strain ANT-03 in treated areas. Since adverse effects were not observed in the mammalian studies, risk to wild mammals is expected to be low.

[1] EPA, 2015. Biopesticides Registration Action Document (BRAD) for *Beauveria bassiana* strain ANT-03. Pesticide Chemical (PC) Code: 129990. U.S. Environmental Protection Agency Office of Pesticide Programs Biopesticides and Pollution Prevention Division. March 30, 2015.

b. Aquatic Organisms

Freshwater Fish Test with *Oncorhynchus mykiss* (Rainbow Trout)

This study was conducted to assess the chronic toxicity and pathogenicity of *Beauveria bassiana* strain ANT-03 to *Oncorhynchus mykiss* (Rainbow Trout) in a 30-day static-renewal test. Since the mean survival rates were not significantly different and no symptomology observed; there were no toxic or pathogenic effects observed (refer to Attachment 16; document 502).

EPA Summary from BRAD (EPA, 2015)

A guideline study submitted for the non-target freshwater fish testing requirements indicated no toxicity/pathogenicity of *Beauveria bassiana* ANT-03 to Oncorhynchus mykiss (rainbow trout) when tested at 1 x 10^6 CFU/mL. However, one of the three replicates of the test substance group (10 of the 30 fish in that group) was not included in the mortality calculations. The study author's explanation for the mortality was that this was likely due to a temporary lack of aeration/drop in dissolved oxygen in the test containers, but no other details (e.g., analysis of the dead fish for the MPCA and/or necropsy) were provided.

Freshwater Aquatic Invertebrate with *Daphnia magna*

This study was conducted to assess the chronic toxicity of the *Beauveria bassiana* strain ANT-03, to the mortality, reproduction and growth of the freshwater invertebrate, *Daphnia magna,* in a 21-day static renewal test. No significant adverse effects were observed in organisms treated with sterile filtrate. Results presented are based on nominal concentrations (refer to Attachment 17; document 506).

EPA Summary from BRAD (EPA, 2015)

A guideline study for the non-target freshwater invertebrate testing showed toxicity to Daphnia magna at 1 x 10^6 CFU/mL. Mortality reached 100% in the 1 x 10^6 test substance group (*Beauveria bassiana* strain ANT-03) by Day 8 and 20% in the 1 x 10^3 test substance group in 21 days. The study is classified as supplemental for the following reasons: The study indicates that *Beauveria bassiana* strain ANT-03 is toxic to Daphnia when tested at 1 x 10^6 conidia/mL. The study describes the EC_{50} values for survival, reproduction and biomass. However, the significance of these EC_{50} values are uncertain, because the study was not designed properly to calculate a definitive EC_{50}. In the daphnia study, two concentrations were tested at 10^3 and 10^6 CFU/mL. Since 100% mortality was observed at the high rate and 20% mortality was observed at the low rate, it can be concluded that the LC_{50} is >10^3 CFU/mL. More testing at

concentrations lower than 10^6 CFU/mL but higher than 10^3 CFU/mL is needed to support the EC_{50} values presented in the Daphnia study.

c. Non-Target Insects and Honey Bees

Arthropods

Ladybird Beetle Non-Target Insect Microbial Testing

EPA Summary from BRAD (EPA, 2015)

The guideline study submitted to meet the requirement for non-target insect testing showed no toxicity to ladybird beetles (Hippodamia convergens) upon a 10-day dietary exposure to corn earworm (Helicoverpa zea) sprayed with *Beauveria bassiana* ANT-03 at 1 x 10^6 CFU/mL. However, reduced food consumption was observed with the test substance group during Days 7- 11 when compared to the untreated control group. It is not possible to determine potential toxicity or pathogenicity in this study, since reduced food consumption could be the result of toxicity/pathogenicity, or could have resulted in reduced exposure during the study. Therefore, these results are inconclusive (refer to Attachment 18; document 503).

Green Lacewing Microbial Testing

EPA Summary from BRAD (EPA, 2015)

The results with green lacewing larvae indicated no significant differences in mortality among different test groups when exposed to *Beauveria bassiana* ANT-03 at 1 x 10^6 CFU/mL in a 25-day dietary toxicity/pathogenicity study. However, the mean number of eggs consumed per day during Days 0-7 in the untreated control group, inactivated test substance group, and test substance group was 19.3, 14.5, and 16.3, respectively. The inactivated test substance group and the test substance group consumed significantly fewer eggs during Days 0-7 than did the untreated control group ($p<0.0001$). (The presence or absence of the surfactant in the various test groups was not clear. Upon request for clarification, the applicant submitted additional information that stated that only test substance and inactive test substance groups included the surfactant but not the untreated control. Surfactants can cause adverse effects in some insects.) The reduced egg consumption observed might be due to the surfactant, but this cannot be confirmed without having a control group treated with the surfactant alone. Without this control, results are inconclusive (refer to Attachment 19; document 504).

Honey bees

The study presents the results of the Semi-Field study trials to evaluate the short and long term impacts of BioCeres WP treatments on honeybee's, *Apis mellifera,* heath and behaviour. In [ ] light of this study, the BioCeres, containing the active ingredient of conidiospores of the strain *B. bassiana* ANT-03, at the highest recommended doses did not infect, cause sub lethal behavioural abnormalities, nor kill the honey bees under field conditions. The current study results indicate that *B. bassiana* strain ANT-03 is safe to honeybees under field conditions (refer to Attachment 20; document 505).

In light of the observation of reduced food consumption in adult ladybird beetles tested and the fact that *Beauveria bassiana* is an entomopathogen known to have a wide host range, the EPA cannot conclude that adverse effects to non-target insects will not occur. It should be best to exercise caution with the uses of the new active ingredient: e.g., to avoid the use of the new active ingredient on or around the crops that are pollinated by insects. The language in the Use Directions on the label under the heading "SENSITIVE AREAS" states, "[t]he pesticide should only be applied when the potential for drift to adjacent sensitive areas (e.g., residential areas, bodies of water, known habitat for threatened or endangered species, non-target crops) is minimal (e.g., when wind is blowing away from the sensitive areas)," which may serve to reduce exposure. Additional language will be required to alert users to the potential for adverse effects to insects, including beneficial species.

A study with honey bees was not provided to support the honey bee data requirement for the proposed FIFRA Section 3 registration of *Beauveria bassiana* strain ANT-03. A data waiver was requested with rationale that included a reference (Al-mazra'awi, 2006) that involved honey bees as a vector for the dispersal of another strain of Beauveria bassiana for control of tarnished plant bug on canola, without observable impact on honey bees. Given that Beauveria bassiana species is a known insect pathogen and honey bee is a major pollinating insect that plays an important role agriculturally as well as environmentally, the absence of the required Tier 1 Honey Bee study is likely to increase concerns about the effects of Beauveria bassiana ANT-03 on honey bees. In addition, the existing data may not be sufficient to predict the effects of Beauveria bassiana strain ANT-03, a unique strain, on other beneficial non-target insects. A vector technology has been developed to use honey bees to disseminate conidia of Beauveria bassiana to control target pests such as whiteflies and thrips (Shipp, 2009), but studies like this generally are not intended to study the direct effects of Beauveria bassiana on honey bees or the bee hives, but to evaluate the effectiveness of honey bee as a vector to transfer the microbial pest control agent. One study (Al Mazra'awi et al., 2007) did examine the effects of another strain of Beauveria bassiana on honey bees. In this study, mortality was low and not significantly different from that of the

controls; however, mycosis in bee cadavers from Beauveria bassiana treated hives indicated the potential for Beauveria bassiana to cause mortality in honey bees.

Based on the above information, the rationale provided by the applicant is not sufficient to determine conclusively that Beauveria bassiana strain ANT-03 will not adversely affect honey bees or that applications to insect pollinated crops will not cause bee mortality. Thus, a statement is required to be added to caution users about the potential effects on bees and other pollinators, and specifically restrict users from applying the end-use product when bees are visiting crops and surrounding areas. This language is likely to reduce exposure, but may not eliminate it completely.

d. Terrestrial plants

Bioceres WP is not phytotoxic.

EPA Summary from BRAD (EPA, 2015)

A guideline study for Non-target Plant Testing, Tier 1 was not submitted, but a data waiver was requested. Scientific rationale submitted for a waiver of the requirement for Non-target Plant Testing consists of the following: *Beauveria bassiana* strain ANT-03 is a naturally occurring soil fungus whose level in the environment would not significantly increase with the registration of the new active ingredient, and the uses of the new a.i. on agricultural crop, turf, ornamental and landscape, and home and garden use sites is not expected to result in increased exposure to non-target plants.

Beauveria bassiana strain ANT-03 is a newly isolated strain from a dead insect in Canada, with new uses (e.g., for terrestrial and food). This strain was stated to be not phytotoxic to certain crops in research and development, but data were not provided to support the claim of no phytotoxicity. Scientific rationale based solely on the published information on existing strains is not sufficient for the waiver request for the new a.i. However, while *Beauveria bassiana* is a known entomopathogen, it is generally not known as a plant pathogen. Therefore, the proposed uses of B*eauveria bassiana* ANT-03 do not exceed the EPA's level of concern.

III. Summary of Proposed Amendments to Chapter 4-71A Plant and Non-Domestic Animal Quarantine, Microorganism Import Rules

Dr. Martin Nadeau of Anatis Bioprotection Inc. proposes the following amendments to various lists in Chapter 4-71A, HAR to make the following changes:

1. Section 4-71A-21A, HAR, List of Restricted Microorganisms (Part A).

   In the Fungi section, amends the existing listing of "*Beauveria* – all species in genus" to read "*Beauveria* – all species in genus except: *B. bassiana* strain ANT-03"

2. Section 4-71A-24, HAR, List of Nonrestricted Microorganisms.

   In the Fungi section, adds Scientific Name: "*Beauveria bassiana* strain ANT – 03".

IV. Advisory Subcommittee Review

This request was submitted to the Advisory Subcommittee on Fungi for their review. Their recommendations and comments are as follows

1. I recommend Approval ___ / ___ Disapproval to change the list placement of *Beauveria bassiana* strain ANT-03, a fungi on the List of Restricted Microorganisms (Part A), for future placement on the List of Nonrestricted Microorganisms, for use in a Microbial Bio-Remediation Product.

Dr. Edward Desmond: Recommends approval.

Comments: "Recommendation is contingent upon the ability to conduct bee count. To ensure customers are adhering to labeling and bees are not adversely affected."

Mr. David Clements: Recommends approval.

Comments: "Since other strains of *Beauveria bassiana* have been registered as microbial product in Hawaii and additional strains have been registered by EPA, would it be reasonable to place all 6 of the EPA registered strains on the List of Non-restricted Microorganisms? This would avoid additional reviews of the same nature and create continuity in the listings of *B. bassiana*."

Dr. Susan Schenck: Recommends approval.

Comments: "Since this is a commercial product that has been tested and shown to be safe for honey bees, I recommend approval."

Dr. A. Christian Whelen: Recommends approval.

> Comments: "Recommendation is contingent upon the ability to conduct bee counts to ensure customers are adhering to labeling and bees are not adversely affected."

Dr. Stephen Ferreira: Recommends approval.

> Comments: "For *Beauveria bassiana*, in general my opinion is that it is not a strong or aggressive bioagent. Thus, moving the species to the non-restricted list has my support. My concern would be the potential for non-target interactions with bees. While the applicant has addressed this concern, I don't think the studies are conclusive. Any use of this biocide should be made with the view of minimizing non-target impacts on bees. I would support moving *B. bassiana* to the non-restricted list, but I would council that the entire species be reclassified, not the specific strain being requested. Either we approve B. bassiana across the board or we don't allow individual exceptions, or we risk future reviews on a case-by-case basis, which I think is not warranted for *B. bassiana*."

Dr. Raquel Wong: Recommends approval.

> Comments: None.

Dr. George Wong: Recommends disapproval.

> Comments: "I have reservations concerning changing *Beauveria bassiana* to placement as a nonrestricted microorganism. A question came up as to why this species is even on the restricted list. I think that can be best answered by saying that there are concerns as to preserving even native insects in Hawaii. Something that other parts of the United States don't share. On the mainland, various strains of B. bassiana are authorized for commercial use to rid insect pests. Obviously, these strains cannot guarantee that only listed species will be targeted, but since they are not concerned with endangered insects, that problem is not considered, but we have insect species that are on the endangered list. I know that it has been tested for honeybees, but what about other insects. There are lots of native insects and you can't test them all!"

ADVISORY COMMITTEE REVIEW: May we request your recommendation and comments at the next meeting of the Advisory Committee on Plants and Animals.

January 19, 2024. At the request of ANATIS BIOPROTECTION's President Silvia Todorova, attachments 2, 3,14,16,17,18,and 19 were considered confidential and has requested that HDOA remove the attachments from the HDOA public access website.

FOR ORGANIC PRODUCTION

Attachment 1

BioCeres WP

OMRI LISTED For Organic Use

KEEP OUT OF REACH OF CHILDREN

CAUTION

Si usted no entiende la etiqueta, busque a alguien para que se la explique a usted en detalle. (If you do not understand this label, find someone to explain it to you in detail.)

FIRST AID
IF IN EYES: Hold eye open and rinse slowly and gently with water for 15–20 minutes. Remove contact lenses, if present, after the first 5 minutes, then continue rinsing eye. Call a poison control center or doctor for treatment advice.
IF INHALED: Move person to fresh air. If person is not breathing, call 911 or an ambulance, then give artificial respiration, preferably by mouth-to-mouth if possible. Call a poison control center or doctor for treatment advice.
IF SWALLOWED: Call a poison control center or doctor immediately for treatment advice. Have person sip a glass of water if able to swallow. Do not induce vomiting unless told to do so by the poison control center or doctor. Do not give anything by mouth to an unconscious person.
HOTLINE NUMBER: Have the product container or label with you when calling a poison control center or doctor, or going for treatment. You may also contact 1-800-222-1222 for emergency medical treatment information.

See inside booklet for additional precautionary statements and directions for use.

1610-0

Biological Mycoinsecticide

Wettable Powder

ACTIVE INGREDIENT

Beauveria bassiana strain ANT-03*	20.0%
OTHER INGREDIENTS:	80.0%
TOTAL:	100.0%

*Contains a minimum of 1 X 10^{10} viable conidia/gram of product.

Manufactured for: Anatis Bioprotection Inc.
278, rang Saint-André, St-Jacques-le-Mineur, Québec J0J 1Z0, Canada

EPA Registration No. 89600-2
EPA Establishment No. 92957-MI-001

BioSafe Systems

Distributed by: BioSafe Systems, LLC
22 Meadow St., East Hartford, CT 06108

NET CONTENTS: 1 lb.

V2
041320

PRECAUTIONARY STATEMENTS HAZARDS TO HUMANS AND DOMESTIC ANIMALS

CAUTION: Causes moderate eye irritation. Harmful if inhaled or swallowed. Avoid contact with eyes or clothing. Avoid breathing dust or spray mist. Wash thoroughly with soap and water after handling and before eating, drinking, chewing gum, using tobacco or using the toilet. Remove and wash contaminated clothing before reuse.

PERSONAL PROTECTIVE EQUIPMENT (PPE)

Applicators and other handlers must wear:

- long-sleeved shirt and long pants
- waterproof gloves
- protective eyewear
- shoes plus socks

Mixer/loaders and applicators must wear a NIOSH-approved particulate respirator with any N, R, or P filter with NIOSH approval number prefix TC-84A; or a NIOSH-approved powered air purifying respirator with an HE filter with NIOSH approval number prefix TC-21C. Repeated exposure to high concentrations of microbial proteins can cause allergic sensitization.

Follow the manufacturer's instructions for cleaning/maintaining PPE. If no such instructions for washables are available, use detergent and hot water. Keep and wash PPE separately from other laundry.

ENGINEERING CONTROLS: When handlers use closed systems, enclosed cabs or aircraft in a manner that meets the requirements listed in the Worker Protection Standard (WPS) for agricultural pesticides (40 CFR 170.607 (d) and (e) (f) for aerial application), the handler PPE requirements may be reduced or modified as specified in the WPS.

IMPORTANT: When reduced PPE is worn because a closed system is being used, handlers must be provided all PPE specified above for "applicators and other handlers" and have such PPE immediately available for use in an emergency, such as a spill or equipment breakdown.

USER SAFETY RECOMMENDATIONS

Users should:

- Remove clothing/PPE immediately if pesticide gets inside. Then wash thoroughly and put on clean clothing.
- Remove PPE immediately after handling this product. Wash the outside of gloves before removing. As soon as possible, wash thoroughly and change into clean clothing.

ENVIRONMENTAL HAZARDS: For terrestrial uses - This product may harm beneficial insects and honey bees. Do not apply while bees or other pollinating insects are actively foraging. This product may be harmful to aquatic organisms. Drift and runoff may be hazardous to aquatic organisms in water adjacent to treated areas. Do not apply within 50 feet of aquatic habitats (such as, but not limited to, lakes, reservoirs, rivers, streams, marshes, ponds, estuaries, and commercial fish ponds). Do not apply directly to water, or to areas where surface water is present or to intertidal areas below the mean high water mark. Do not contaminate water when disposing of equipment washwater or rinsate.

DIRECTIONS FOR USE

It is a violation of Federal law to use this product in a manner inconsistent with its labeling. For any requirements specific to your State or Tribe, consult the State or Tribal agency responsible for pesticide regulation. Do not apply this product in a way that will contact workers or other persons, either directly or through drift. Only protected handlers may be in the area during application.

Agricultural Use Requirements

Use this product only in accordance with its labeling and with the Worker Protection Standard, 40 CFR Part 170. This Standard contains requirements for the protection of agricultural workers on farms, forests, nurseries, and greenhouses, and handlers of agricultural pesticides. It contains requirements for training, decontamination, notification, and emergency assistance. It also contains specific instructions and exceptions pertaining to the statements on this label about personal protective equipment (PPE) and restricted-entry interval. The requirements in this box only apply to uses of this product that are covered by the Worker Protection Standard. Do not enter or allow worker entry into treated areas during the restricted-entry interval (REI) of 4 hours.

PPE required for early entry to treated areas (that is permitted under the Worker Protection Standard and that involves contact with anything that has been treated, such as plants, soil or water) is:

- Coveralls
- Waterproof gloves
- Shoes plus socks

EXCEPTION: If the product is soil incorporated or soil injected, the Worker Protection Standard, under certain circumstances, allows workers to enter the treated area if there will be no contact with anything that has been treated.

NON-AGRICULTURAL USE REQUIREMENTS

The requirements in this box apply to uses of this product that are not within the scope of the Worker Protection Standard for agricultural pesticides (40 CFR Part 170). The WPS applies when this product is used to produce agricultural plants on farms, forests, nurseries or greenhouses.

Keep unprotected persons out of treated areas until sprays have dried.

PRODUCT INFORMATION

Bioceres WP is a biological insecticide containing the active ingredient *Beauveria bassiana* strain ANT-03 for use on labeled growing crops to control insect pests. Bioceres WP acts as a contact biological insecticide for use in the control or suppression of labeled foliar-feeding pests, including aphids, white flies, thrips, plant bugs, beetles and weevils infesting labeled crops or use sites. Bioceres WP must be mixed with water and applied as a foliar spray with ground or aerial equipment equipped for conventional insecticide spraying, or by chemigation, in field or greenhouse use sites.

PRE-HARVEST INTERVAL (PHI): Pre-harvest interval for Bioceres WP is zero (0) days. Bioceres WP can be applied up to the day of harvest.

USE INSTRUCTIONS

Bioceres WP is a selective insecticide for use against labeled insects. Close scouting and early attention to infestations is highly recommended. Proper timing of application targeting newly hatched larvae is important for optimal results.

Thorough coverage of infested plant parts is necessary for effective control. Bioceres WP does not have systemic activity. For some crops, directed drop nozzles by ground machine are required. Under heavy pest populations, use the stated higher label rates, shorten the spray interval, and/or increase the spray volume to improve coverage. Repeat applications at an interval sufficient to maintain control, usually 3-10 days depending upon plant growth rate, insect activity, and other factors. If attempting to control an insect population with a single application, make the treatment when eggs start hatching, but before economic damage occurs.

To enhance control, tank mix with contact insecticides/miticides/nematicides. Use the lower label rates of Bioceres WP when populations are low and when tank-mixing with other insecticides/miticides/nematicides. Use the stated higher rates of Bioceres WP when applied standalone, when populations are high or when egg numbers are high.

To enhance adhesion of Bioceres WP use a spreader/sticker adjuvant.

Bioceres WP has been evaluated for phytotoxicity on a variety of crops under various normal growing conditions. However, testing all crop varieties, in all mixtures and combinations, is not feasible. Prior to treating entire crop, test a small portion of the crop for sensitivity.

GROUND AND AERIAL APPLICATIONS

Apply Bioceres WP in ground and aerial equipment with quantities of water sufficient to provide thorough coverage of infested plant parts. The amount of water needed per acre will depend upon crop development, weather, application equipment, and local experience. Do not spray when wind speed favors drift beyond the area intended for use. Avoiding spray drift is the responsibility of the applicator.

Mixing directions

Important – Do not add Bioceres WP to the mix tank before introducing the correct amount of water. Add water to the mix tank. Start the mechani-

cal or hydraulic agitation to provide moderate circulation before adding Bioceres WP. Add spreader/sticker and then correct amount of Bioceres WP to the mix tank and continue circulation. Maintain circulation while loading and spraying. Do not mix more Bioceres WP than can be used in 24 hours.

Spray volume

For conventional air and ground applications, use at least 50 gallons of total volume per acre in water-based sprays.

Tank mixing

Do not tank mix with fungicides. Do not combine Bioceres WP in the spray tank with other pesticides, surfactants, adjuvants, or fertilizers if there has been no previous experience or use of the combination to show it is physically compatible, effective, and non-injurious under your use conditions. Observe the most restrictive of the labeling limitations and precautions of all products used in mixtures.

To ensure compatibility of tank-mix combinations, they must be evaluated prior to use. To determine the physical compatibility of this product with other products, use a jar test. Using a quart jar, add the proportionate amounts of the products to one quart of water with agitation. Add dry formulations first, then flowables, and then emulsifiable concentrates last. After thoroughly mixing, let this mixture stand for 5 minutes. If the combination remains mixed or can be readily remixed, it is physically compatible. Once compatibility has been proven, use the same procedure for adding required ingredients to the spray tank.

AERIAL DRIFT REDUCTION INFORMATION

GENERAL: Avoiding spray drift at the application site is the responsibility of the applicator and the grower (specifically, see **SENSITIVE AREAS** section for the requirement regarding spray drift and honey bees). The interaction of many equipment- and weather-related factors determine the potential for spray drift. The applicator and the grower are responsible for considering all these factors when making decisions. Where states have more stringent regulations, they must be observed.

Do not apply directly to aquatic habitats (such as, but not limited to, lakes, reservoirs, rivers, streams, marshes, ponds, estuaries, and commercial fish ponds).

INFORMATION ON DROPLET SIZE: Use only medium or coarser spray nozzles according to ASAE (S572) definition for standard nozzles. In conditions of low humidity and high temperatures, applicators should use a coarser droplet size. The most effective way to reduce drift potential is to apply large droplets. The best drift management strategy is to apply the largest droplets that will provide sufficient coverage and control. Applying larger droplets reduces drift potential, but will not prevent drift if applications are made improperly, or under unfavorable environmental conditions (see Wind, Temperature and Humidity, and Temperature Inversions).

CONTROLLING DROPLET SIZE: Volume – Use high flow rate nozzles to apply the highest practical spray volume. Nozzles with higher rated flows produce larger droplets. Pressure – Do not exceed the nozzle manufacturer's specified pressures. For many nozzle types, lower pressure produces larger droplets. When high flow rates are needed, use higher flow rate nozzles instead of increasing pressure. Number of Nozzles – Use the minimum number of nozzles that provide uniform coverage. Nozzle Orientation – Orienting nozzles so that the spray is released parallel to the airstream produces larger droplets than other orientations and is the recommended practice. Significant deflection from horizontal will reduce droplet size and increase drift potential. Nozzle Type – Use a nozzle type that is designed for the intended application. With most nozzle types, narrower spray angles produce larger droplets. Consider using low-drift nozzles. Solid stream nozzles oriented straight back produce the largest droplets and the lowest drift.

BOOM WIDTH: For aerial applications, the boom width must not exceed 75% of the wingspan or 90% of the rotary blade.

APPLICATION HEIGHT: Do not make application at a height greater than 10 feet above the top of the largest plants unless a greater height is required for aircraft safety. Making applications at the lowest height that is safe reduces exposure to droplets to evaporation and wind. If application includes a no-spray zone, do not release spray at a height greater than 10 feet above the ground or crop canopy.

SWATH ADJUSTMENT: When applications are made with a crosswind, the swath will be displaced downward. Therefore, on the up and downwind edges of the field, the applicator must compensate for this displacement by adjusting the path of the aircraft upwind. Swath adjustment distance should increase with increasing drift potential (higher wind, smaller drops, etc.).

WIND: Only apply this product if the wind direction favors on-target deposition. Do not apply when the wind velocity exceeds 15 mph. Drift potential is lowest between wind speeds of 2-10 mph. However, many factors, including droplet size and equipment type determine drift potential at anygiven speed. Application should be avoided below 2 mph due to variable wind direction and high inversion potential. NOTE: Local terrain can influence wind patterns. Every applicator should be familiar with local wind patterns and how they affect spray drift.

TEMPERATURE AND HUMIDITY: When making applications in low relative humidity, set up equipment to produce larger droplets to compensate for evaporation. Droplet evaporation is most severe when conditions are both hot and dry.

TEMPERATURE INVERSIONS: Do not apply during a temperature inversion because drift potential is high. Temperature inversions restrict vertical air mixing, which causes small, suspended droplets to remain in a concentrated cloud. This cloud can move in unpredictable directions due to the light variable winds common during inversions. Temperature inversions are characterized by increasing temperatures with altitude and are common on nights with limited cloud cover and light to no wind. They begin to form as the sun sets and often continue into the morning. Their presence can be indicated by ground fog; however, if fog is not present, inversions can also be identified by the movement of smoke from a ground source or an aircraft smoke generator. Smoke that layers and moves laterally in a concentrated cloud (under low wind conditions) indicates an inversion, while smoke that moves upward and rapidly dissipates indicates good vertical air mixing.

SENSITIVE AREAS: The pesticide should only be applied when the potential for drift to adjacent sensitive areas (e.g. residential areas, bodies of water, known habitat for threatened or endangered species, non-target crops) is minimal (e.g. when wind is blowing away from the sensitive areas). Do not allow spray to drift from the application site and contact people, structures people occupy at any time and the associated property, parks and recreation areas, non-target crops, blooming crops or weeds that bees are visiting, aquatic and wetland areas, woodlands, pastures, rangelands, or animals.

CHEMIGATION USE DIRECTIONS

Spray preparation

First, prepare a suspension of Bioceres WP in a mix tank. Fill tank ½-¾ of the amount of water for the area to be treated. Start mechanical or hydraulic agitation. Add the required amount of Bioceres WP, and then the remaining volume of water. Then set the sprinkler to deliver a minimum of 0.1-0.3 inch of water per acre. Start sprinkler and uniformly inject the suspension of Bioceres WP into the irrigation water line so as to deliver the correct rate of Bioceres WP per acre. Inject the suspension of Bioceres WP with a positive displacement pump into the main line ahead of a right angle turn to ensure adequate mixing. Bioceres WP is to be metered continuously for the duration of the water application. If you have questions about calibration, you should contact State Extension Service specialists, equipment manufacturers or other experts.

Do not combine Bioceres WP with other pesticides, surfactants, adjuvants, or fertilizers for application through chemigation equipment unless prior experience has shown the combination to be physically compatible, effective and non-injurious under conditions of use.

General Requirements -

1. Apply this product only through sprinkler, including center pivot, lateral move, end tow, side (wheel) roll, traveler, big gun, solid set, or hand move, or drip (trickle) irrigation systems. Do not apply this product through any other type of irrigation system.

2. Crop injury, lack of effectiveness, or illegal pesticide residues in the crop can result from non-uniform distribution of treated water.
3. If you have questions about calibration, you should contact State Extension Service specialists, equipment manufacturers or other experts.
4. Do not connect an irrigation system (including greenhouse systems) used for pesticide application to a public water system unless the pesticide label-prescribed safety devices for public water systems are in place.
5. A person knowledgeable of the chemigation system and responsible for its operation, or under the supervision of the responsible person, shall shut the system down and make necessary adjustments should the need arise.

Specific Requirements for Chemigation Systems Connected to Public Water Systems -

1. Public water system means a system for the provision to the public of piped water for human consumption if such system has at least 15 service connections or regularly serves an average of at least 25 individuals daily at least 60 days out of the year.
2. Chemigation systems connected to public water systems must contain a functional, reduced-pressure zone, backflow preventer (RPZ) or the functional equivalent in the water supply line upstream from the point of pesticide introduction. As an option to the RPZ, the water from the public water system should be discharged into a reservoir tank prior to pesticide introduction. There shall be a complete physical break (air gap) between the flow outlet end of the fill pipe and the top or overflow rim of the reservoir tank of at least twice the inside diameter of the fill pipe.
3. The pesticide injection pipeline must contain a functional, automatic, quick-closing check valve to prevent the flow of fluid back toward the injection pump.
4. The pesticide injection pipeline must contain a functional, normally closed, solenoidoperated valve located on the intake side of the injection pump and connected to the system interlock to prevent fluid from being withdrawn from the supply tank when the irrigation system is either automatically or manually shut down.
5. The system must contain functional interlocking controls to automatically shut off the pesticide injection pump when the water pump motor stops, or in cases where there is no water pump, when the water pressure decreases to the point where pesticide distribution is adversely affected.
6. Systems must use a metering pump, such as a positive displacement injection pump (e.g., diaphragm pump) effectively designed and constructed of materials that are compatible with pesticides and capable of being fitted with a system interlock.
7. Do not apply when wind speed favors drift beyond the area intended for treatment.

Specific Requirements for Sprinkler Chemigation -

1. The system must contain a functional check valve, vacuum relief valve and low-pressure drain appropriately located on the irrigation pipeline to prevent water source contamination from backflow.
2. The pesticide injection pipeline must contain a functional, automatic, quick-closing check valve to prevent the flow of fluid back toward the injection pump.
3. The pesticide injection pipeline must also contain a functional, normally closed, solenoidoperated valve located on the intake side of the injection pump and connected to the system interlock to prevent fluid from being withdrawn from the supply tank when the irrigation system is either automatically or manually shut down.
4. The system must contain functional interlocking controls to automatically shut off the pesticide injection pump when the water pump motor stops.
5. The irrigation line or water pump must include a functional pressure switch, which will stop the water pump motor when the water pressure decreases to the point where pesticide distribution is adversely affected.
6. Systems must use a metering pump, such as a positive displacement injection pump (e.g., diaphragm pump) effectively designed and constructed of materials that are compatible with pesticides and capable of being fitted with a system interlock.

7. Do not apply when wind speed favors drift beyond the area intended for treatment.

Specific Requirements for Drip (Trickle) Chemigation -

1. The system must contain a functional check valve, vacuum relief valve and low-pressure drain appropriately located on the irrigation pipeline to prevent water source contamination from backflow.
2. The pesticide injection pipeline must contain a functional, automatic, quick-closing check valve to prevent the flow of fluid back toward the injection pump.
3. The pesticide injection pipeline must also contain a functional, normally closed, solenoidoperated valve located on the intake side of the injection pump and connected to the system interlock to prevent fluid from being withdrawn from the supply tank when the irrigation system is either automatically or manually shut down.
4. The system must contain functional interlocking controls to automatically shut off the pesticide injection pump when the water pump motor stops.
5. The irrigation line or water pump must include a functional pressure switch which will stop the water pump motor when the water pressure decreases to the point where pesticide distribution is adversely affected.
6. Systems must use a metering pump, such as a positive displacement injection pump (e.g., diaphragm pump) effectively designed and constructed of materials that are compatible with pesticides and capable of being filled with a system interlock.

Application Instructions for All Types of Chemigation -

1. Remove scale, pesticide residues, and other foreign matter from the chemical supply tank and entire injector system. Flush with clean water. Failure to provide a clean tank, void of scale or residues may cause product to lose effectiveness or strength.
2. Determine the treatment rates as indicated in the directions for use and make proper dilutions.
3. Prepare a solution in the chemical tank by filling the tank with the required water and then adding product as required. Utilize agitation to keep solution in suspension.

SOIL APPLICATION RATES

Apply Bioceres WP as a soil drench or injection to treat the below-mentioned pests and crops. For soil applications, apply 1-4 oz. (up to ¼ lb.) of Bioceres WP per 1,000 square feet. For difficult to control soil pests, apply Bioceres WP at the high rate (4 oz. per 1,000 square feet). Do not apply to water-saturated soil.

Apply Bioceres WP in enough water to ensure good coverage of treated area, at least one gallon per 1,000 square feet. Irrigate treated area after application to disperse product into soil.

GREENHOUSE AND OUTDOOR FOLIAR APPLICATION RATES

Apply Bioceres WP to treat the below mentioned pests and crops. Repeat at 5-7 day intervals as needed. Use 50 gallons spray volume per acre. Thoroughly cover plant foliage with spray solution, but not to runoff. Pre-harvest Interval (PHI) = 0 days.

Use a range of 1.5-3.0 lbs. per 100 gallons

Alfalfa (Hay and Seed), Hay and Other Forage Crops

(1-3 pounds of Bioceres WP per acre)
(2-5 briquettes of Bioceres WP per acre)
Aphids and plant bugs

Asparagus

(1-3 pounds of Bioceres WP per acre)
(2-5 briquettes of Bioceres WP per acre)
Whiteflies, aphids, thrips, and plant bugs

BRASSICA (COLE) LEAFY VEGETABLES:
Including, but not limited to (excludes watercress):

Broccoli, Broccoli Raab, Brussels Sprouts, Cabbage, Chinese Broccoli, Chinese Cabbage (Bok Choy), Chinese Cabbage (Napa), Chinese Mustard Cabbage (Gai Choy), Cauliflower, Cavalo Broccolo, Collards, Kale, Kohlrabi, Mizuna, Mustard Greens, Mustard Spinach, and Rape Greens

(1-3 pounds of Bioceres WP per acre)
(2-5 briquettes of Bioceres WP per acre)
Whiteflies, aphids, thrips, and plant bugs

BULB VEGETABLES: Including, but not limited to:

Leek, Garlic, Onion (Bulb and Green), Welch, and Shallot

(1-3 pounds of Bioceres WP per acre)
(2-5 briquettes of Bioceres WP per acre)
Whiteflies, aphids, thrips, and plant bugs

BERRY and SMALL FRUIT:
Including, but not limited to:

Bushberry: Blueberry, Currant, Gooseberry, Huckleberry, Elderberry, Juneberry, Ligonberry, and Salal

(1-2 pounds of Bioceres WP per acre)
(2-3 briquettes of Bioceres WP per acre)
Whiteflies, aphids, thrips, and plant bugs

Caneberry: Blackberry, Loganberry, Red and Black Raspberry, and cultivars, varieties and/or hybrids of these

(1-2 pounds of Bioceres WP per acre)
(2-3 briquettes of Bioceres WP per acre)
Whiteflies, aphids, Spotted wing drosophila (vinegar fly) (*Drosophila Suzukii*), thrips, plant bugs and bud weevil

Grape, Amur River Grape, Gooseberry, Kiwifruit, Maypop, Schisandra Berry

(1-2 pounds of Bioceres WP per acre)
(2-3 briquettes of Bioceres WP per acre)
Whiteflies, aphids, Spotted wing drosiphila (vinegar fly) (*Drosophila Suzukii*), thrips, and plant bugs

Strawberry
(1-3 pounds of Bioceres WP per acre)
(2-5 briquettes of Bioceres WP per acre)
Whiteflies, aphids, thrips, plant bugs, and bud weevil

CEREAL GRAINS (not including rice):
Including, but not limited to:

Barley, Buckwheat, Oats, Pearl Millet, Proso, Millet, Rye, Sorghum (Milo), Teosine, Triticale, and Wheat

(1-3 pounds of Bioceres WP per acre)
(2-5 briquettes of Bioceres WP per acre)
Whiteflies, aphids and thrips

Corn (Field Corn, Sweet Corn, Popcorn and Corn Grown for Seed)

(1-3 pounds of Bioceres WP per acre)
(2-5 briquettes of Bioceres WP per acre)
Whiteflies, aphids and thrips

CITRUS FRUITS: Including, but not limited to:

Calamondin, Citrus citron, Citrus hybrids (includes Chironja, Tangelo, Tangor), Grapefruit, Kumquat, Lemon, Lime, Mandarin (Tangerine), Orange (sour and sweet), Pummelo, and Satsuma mandarin

(1-3 pounds of Bioceres WP per acre)
(2-5 briquettes of Bioceres WP per acre)
Whiteflies, aphids and thrips

Cotton

(1-3 pounds of Bioceres WP per acre)
(2-5 briquettes of Bioceres WP per acre)
Whiteflies, aphids and thrips

CUCURBIT VEGETABLES:
Including, but not limited to:

Chayote (fruit); Chinese waxgourd (Chinese preserving melon); Citron melon; Cucumber; Gherkin; Gourd, edible (includes Hyotan, Cucuzza, Hechima, Chinese okra); Melons; *Momordica* spp (includes Balsam apple, Balsam pear (bittermelon), Chinese cucumber); Muskmelon (includes Cantaloupe); Pumpkin; Squash (summer and winter) (includes Butternut squash, Calabaza, Hubbard squash, Acorn squash, Spaghetti squash); Watermelon

(1-3 pounds of Bioceres WP per acre)
(2-5 briquettes of Bioceres WP per acre)

Whiteflies, aphids, thrips, plant bugs, and striped cucumber beetle

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FRUITING VEGETABLES:
Including, but not limited to:

Tomato, Tomatillo, Pepper, Groundcherry, Pepino, Okra, and Eggplant

(1-3 pounds of Bioceres WP per acre)
(2-5 briquettes of Bioceres WP per acre)
Whiteflies, aphids, thrips, plant bugs, and European corn borer

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HERBS AND SPICES: Including, but not limited to:

Angelica, Balm, Basil, Borage, Burnet, Chamomile, Catnip, Chervil, Chive, Clary, Coriander, Costmary, Cilantro, Curry, Dillweed, Horehound, Hyssop, Lavender, Lemongrass, Lovage, Marjoram, Nasturtium, Parsley (Dried), Rosemary, Sage, Savory (Summer and Winter), Sweet Bay, Tansy, Tarragon, Thyme, Wintergreen, Woodruff and Wormwood

(1-3 pounds of Bioceres WP per acre)
(2-5 briquettes of Bioceres WP per acre)
Whiteflies, aphids, thrips, and plant bugs

Hops and Dried Cones

(1-3 pounds of Bioceres WP per acre)
(2-5 briquettes of Bioceres WP per acre)
Whiteflies, aphids and thrips

LEAFY VEGETABLES (Except BRASSICA Vegetables): Including, but not limited to:

Arugula, Celery, Corn Salad, Cress, Dandelion, Dock, Edible Chrysanthemum, Endive, Fennel, Head Lettuce, Leaf Lettuce, Parsley, Purslane, Radicchio, Rhubarb, Spinach and Swiss Chard

(1-3 pounds of Bioceres WP per acre)
(2-5 briquettes of Bioceres WP per acre)
Whiteflies, aphids, thrips, and plant bugs

LEAVES OF ROOT AND TUBER VEGETABLES:
Including, but not limited to:

Beet and Turnip

(1-3 pounds of Bioceres WP per acre)
(2-5 briquettes of Bioceres WP per acre)
Whiteflies, aphids, thrips, and plant bugs

LEGUME VEGETABLES (SUCCULENT OR DRIED) AND GRAIN CROPS: Including, but not limited to:

Adzuki Bean, Blackeyed Pea, Beans, Chickpea, Cowpea, Crowder Pea, Edible-Pod Pea, English Pea, Fava Bean, Field Bean, Field Pea, Garbanzo Bean, Garden Pea, Green Pea, Kidney Bean, Lentils, Lima Bean, Lupins, Mung Bean, Navy Bean, Peas, Pigeon Pea, Pinto Bean, Runner Bean, Snap Bean, Snow Pea, Soybean, Sugar Snap Pea, Tepary Bean, Wax Bean, and Yardlong Bean

(1-3 pounds of Bioceres WP per acre)
(2-5 briquettes of Bioceres WP per acre)
Whiteflies, aphids, thrips, and plant bugs

OILSEED CROPS: Including, but not limited to:

Canola, Safflower, Sesame, Sunflower (including Sunflower Grown for Seed), Tea, and Jojoba

(1-3 pounds of Bioceres WP per acre)
(2-5 briquettes of Bioceres WP per acre)
Whiteflies, aphids and thrips

Peanut

(1-3 pounds of Bioceres WP per acre)
(2-5 briquettes of Bioceres WP per acre)
Whiteflies, aphids and thrips

POME FRUITS: Including, but not limited to:

Apples, Crabapple, Loquat, Mayhaw, Oriental Pear, Pears and Quince

(1-3 pounds of Bioceres WP per acre)
(2-5 briquettes of Bioceres WP per acre)
Whiteflies, aphids, thrips, and plant bugs

ROOT AND TUBER VEGETABLES:
Including, but not limited to:

Artichoke, Black Salsify, Carrot, Cassava (bitter or sweet), Celeriac (celery root), Chayote (root), Chicory, Chinese Artichoke, Edible Burdock, Garden Beet, Ginger, Ginseng, Horseradish, Jerusalem Artichoke, Oriental Radish, Parsnip, Potatoes, Radish, Rutabaga, Salsify, Skirret, Spanish Salsify, Sugar Beet, Sweet Potatoes, Taro, Tumeric, Turnip, Turniprooted Chervil, Turnip-rooted Parsley and Yams

(1-3 pounds of Bioceres WP per acre)
(2-5 briquettes of Bioceres WP per acre)
Whiteflies, aphids, thrips, and plant bugs

Shade and Ornamental Trees

(1-3 pounds of Bioceres WP per acre)
(2-5 briquettes of Bioceres WP per acre)
Whiteflies, aphids, thrips, and plant bugs

STONE FRUITS: Including, but not limited to:

Apricots, Cherry, Nectarine, Peach, Plum, and Prune

(1-3 pounds of Bioceres WP per acre)
(2-5 briquettes of Bioceres WP per acre)
Whiteflies, aphids, Spotted wing drosophila (vinegar fly) (*Drosophila Suzukii*), thrips, and plant bugs

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TREE NUTS: Including, but not limited to:

Almonds, Cashew, Chestnut, Filbert (Hazelnut), Macadamia Nut, Pecan, Pistachios, and Walnut

(1-3 pounds of Bioceres WP per acre)
(1.5-4.5 briquettes of Bioceres WP per acre)
Whiteflies, aphids and thrips

TROPICAL AND SUBTROPICAL FRUITS:
Including, but not limited to:

Acerola, Atemoya, Avocado, Banana, Biriba, Black Sapote, Canistel, Cherimoya, Custard Apple, Feijoa, Guava, Ilama, Jaboticaba, Kiwi, Longan, Lychee, Mamey Sapote, Mango, Olive, Papaya, Passionfruit, Pineapple, Plantains, Pulasan, Rambutan, Sapodilla, Soursop, Spanish Lime, Star Apple, Starfruit, Sugar Apple, Ti Palm Leaves, Wax Jambu (Wax Apple), and White Sapote

(1-3 pounds of Bioceres WP per acre)
(2-5 briquettes of Bioceres WP per acre)
Whiteflies, aphids, thrips, and plant bugs

Fig

(1-2 pounds of Bioceres WP per acre)
(2-3 briquettes of Bioceres WP per acre)
Whiteflies, aphids, thrips, and plant bugs

ADDITIONAL PLANTS:

Coffee, Hemp, Mushroom, Sugar Cane, and Tobacco

(1-3 pounds of Bioceres WP per acre)
(2-5 briquettes of Bioceres WP per acre)
Whiteflies, aphids, thrips, and plant bugs

FOR USE ON THE FOLLOWING SITES FOR CONTROL OF INSECTS:

Ornamentals in parks and landscapes including: Flowering plants, foliage plants, broadleaves, shrubs, trees, conifers

(1-3 pounds of Bioceres WP per acre)
(2-5 briquettes of Bioceres WP per acre)
Whiteflies, aphids, thrips, and plant bugs

Turfgrasses in parks, landscapes, and golf courses

(3 – 6 pounds of Bioceres WP per acre)
(4.5-9 briquettes of Bioceres WP per acre)
Cinch bugs, white grubs, and plant bugs

STORAGE AND DISPOSAL

Do not contaminate water, food or feed by storage and disposal.

Pesticide Storage: Product can be stored up to 18 months at 39 ± 2°F (4 ± 2°C). Store at refrigerator temperatures in a dry place. Avoid overheating.

Pesticide Disposal: To avoid wastes, use all material in this container by application according to label directions. If wastes cannot be avoided, offer remaining product to a waste disposal facility or pesticide disposal program (often such programs are run by state or local governments or by industry).

Container Handling: Non refillable container. Do not reuse or refill this container. Completely empty bag into application equipment. Then offer for recycling if available, or dispose of empty bag in a sanitary landfill or by incineration. Do not burn, unless allowed by state and local ordinances. (For instances where state and local ordinances do allow burning): If burned, stay out of smoke.

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See inside booklet for additional precautionary statements and directions for use.

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ACTIVE INGREDIENT

Beauveria bassiana strain ANT-03*.....	20.0%
OTHER INGREDIENTS:.....	80.0%
TOTAL:.....	100.0%

*Contains a minimum of 1 X 10^{10} viable conidia/gram of product.

PRECAUTIONARY STATEMENTS
HAZARDS TO HUMANS AND DOMESTIC ANIMALS

CAUTION: Causes moderate eye irritation. Harmful if inhaled or swallowed. Avoid contact with eyes or clothing. Avoid breathing dust or spray mist. Wash thoroughly with soap and water after handling and before eating, drinking, chewing gum,using tobacco or using the toilet. Remove and wash contaminated clothing before reuse.

FIRST AID

IF IN EYES: Hold eye open and rinse slowly and gently with water for 15–20 minutes. Remove contact lenses, if present, after the first 5 minutes, then continue rinsing eye. Call a poison control center or doctor for treatment advice.

IF INHALED: Move person to fresh air. If person is not breathing, call 911 or an ambulance, then give artificial respiration, preferably by mouth-to-mouth if possible. Call a poison control center or doctor for treatment advice.

IF SWALLOWED: Call a poison control center or doctor immediately for treatment advice. Have person sip a glass of water if able to swallow. Do not induce vomiting unless told to do so by the poison control center or doctor. Do not give anything by mouth to an unconscious person.

HOTLINE NUMBER: Have the product container or label with you when calling a poison control center or doctor, or going for treatment. You may also contact 1-800-222-1222 for emergency medical treatment information.

Manufactured for: Anatis Bioprotection Inc.
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EPA Establishment No. 92957-MI-001

KEEP OUT OF REACH OF CHILDREN

CAUTION

Si usted no entiende la etiqueta, busque a alguien para que se la explique a usted en detalle.
(If you do not understand this label, find someone to explain it to you in detail.)

STORAGE AND DISPOSAL

Do not contaminate water, food or feed by storage and disposal.

Pesticide Storage: Product can be stored up to 18 months at 39 ± 2°F (4 ± 2°C). Store at refrigerator temperatures in a dry place. Avoid overheating.

Pesticide Disposal: To avoid wastes, use all material in this container by application according to label directions. If wastes cannot be avoided, offer remaining product to a waste disposal facility or pesticide disposal program (often such programs are run by state or local governments or by industry).

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A study of host specificity in the entomopathogenic fungus *Beauveria bassiana* (Hypocreales, Clavicipitaceae)

K. Uma Devi [a]; J. Padmavathi [a]; C. Uma Maheswara Rao [b]; Akbar Ali P. Khan [c]; Murali C. Mohan [d]
[a] Department of Botany, Andhra University, Visakhapatnam, India [b] Innovation Labs, Advanced Technology Center, Tata Consultancy Services, Tata Consultancy Services, Hyderabad, India [c] Centre for Cellular and Molecular Biology, Hyderabad, India [d] Department of Biotechnology, Gandhi Institute of Technology and Management, Visakhapatnam, India

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A study of host specificity in the entomopathogenic fungus *Beauveria bassiana* (Hypocreales, Clavicipitaceae)

K. Uma Devi [a]*, J. Padmavathi[a], C. Uma Maheswara Rao[b], Akbar Ali P. Khan[c] and Murali C. Mohan[d]

[a]*Department of Botany, Andhra University, Visakhapatnam, India;* [b]*Innovation Labs, Advanced Technology Center, Tata Consultancy Services, Tata Consultancy Services, Hyderabad, India;* [c]*Centre for Cellular and Molecular Biology, Hyderabad, India;* [d]*Department of Biotechnology, Gandhi Institute of Technology and Management, Visakhapatnam, India*

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Beauveria bassiana (Balsamo – Crivelli) Vuillemin based mycoinsecticides are used against agricultural, veterinary and medical insect pests. The fungus has a very diverse and extensive host range. Variation in virulence among isolates of *B. bassiana* to different insect species has been abundantly documented. Given the effect of multiple factors on virulence, it is not certain whether the observed difference in virulence can be labelled as host specificity. Environmental conditions and susceptibility of the insect population are two main factors that affect successful fungal infection. Keeping the environmental factors constant, if virulence of an isolate to different insect species and different populations within an insect species is compared, the scale of difference between the two responses can be estimated. If differences in virulence of an isolate to different insect species are greater than the difference in virulence to different insect populations within an insect species, then, the isolate can be considered as exhibiting specific preference to those insect species towards which it exhibits high virulence. To examine this feature, a worldwide sample of *B. bassiana* was bioassayed on nine insect species and two different populations within two insect species. Laboratory bioassays were done on: *Bombyx mori* (Lepidoptera), *Spodoptera litura* (Lepidoptera), *Chilo partellus* (Lepidoptera), *Helicoverpa armigera* (Lepidoptera), *Epilachna vigintioctopunctata* (Coleoptera), *Mylabris pustulata* (Coleoptera), *Aphis craccivora* (Homoptera), *Maconellicoccus hirsutus* (Hemiptera) and *Oecophylla smaragdina* (Hymenoptera). The range of variation in virulence of a *B. bassiana* isolate to different insect species was not more than that observed with different populations within a single insect species. *B. bassiana* is thus a generalist with no strict host preference. *B. bassiana* based biopesticide can be used as a broad spectrum insecticide against a myriad of insect pests.

Keywords: *Beauveria bassiana*; host specificity; insect populations; insect species; laboratory bioassays; virulence

Introduction

Among the entomopathogenic fungi, *Beauveria bassiana*, a popular registered mycoinsecticide, has an extremely large host list of ~700 insect species (Li 1988; Glare and Milner 1991; Humber 1991; Goettel, Inglis, and Wraight 2000). It is ubiquitous in distribution and is pathogenic to a wide spectrum of arthropods with its host range spanning most orders of class Insecta (Butt and Goettel 2000; Lacey, Frutos, Kaya, and Vial 2001; Zimmerman 2007).

*Corresponding author. Email: umadevikoduru@gmail.com

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It also has the ability to colonise plants (e.g. corn), though not as a pathogen, but as an endophyte (Wagner and Lewis 2000). It has been reported as an endophyte in a few other plants as well (Zimmerman 2007). *Beauveria bassiana* was speculated to contain a diverse assemblage of genotypes and most likely to be a species complex (Goettel et al. 2000). This view has been amply substantiated by molecular genetic data (Rehner and Buckley 2003, 2005; Uma Devi, Reineke, Nageswara Rao Reddy, Uma Maheswara Rao, and Padmavathi 2006). Goettel et al. (2000) envisaged that it will not be surprising to find within the taxon of *B. bassiana* which collectively possesses a very wide host range, individual isolates or pathotypes, with a substantially restricted host range. Fire ants (*Solenopsis invicta*) across South America were found to be infected by a single genotype of *B. bassiana* (Zeng 1999). Isolates of *B. bassiana* infecting European corn borer moth (*Ostrinia nubilalis*) were all found to have identical telomere fingerprints; similar was the case with isolates from sugar cane borer (*Diatraea saccharalis*) (Viaud, Couteaudier, Levis, and Riba 1996; Maurer, Couteaudier, Girard, Bridge, and Riba 1997). *B. bassiana* isolates pathogenic to rice water weevil, *Lissorhoptrus oryzophilus*, were found to have a very similar genotype (Urtz and Rice 1997). Reviews of literature on *B. bassiana* hint at the prevalence of host specificity with strains differing in their host range (Fargues and Remaudiére 1977; Ferron 1978; McCoy 1990; Glare and Milner 1991). Commercial formulations of this fungus have also been developed with isolates specifically virulent against target pests: *Ostrinil*® for corn borer (*Ostrinia nubilalis*) and Boverin for potato beetle (*Leptinotarsa decemlineata*) (Riba, Couteaudier, Maurer, and Neuvéglise 1994). Recommendations have been made to test the virulence of *B. bassiana* isolate to be incorporated in the mycopesticide formulation against the insect to which it is targeted (Ferron, Fargues, and Riba 1991). On the other hand, in a study of the host range of *B. bassiana* using eight lepidopteran insect species (Wraight et al. 2003), all the 40 isolates tested were reported to be pathogenic to all the insect species with numerous isolates being highly virulent to all the tested insects. Naturalis®, a registered *B. bassiana* based mycopesticide formulation was found effective against taxonomically diverse insect pests (Butt, Jackson, and Magan 2001). Naturalis® was labelled as not being effective against lepidopteran pests but was found virulent to *Plutella xylostella*, a lepidopteran (Vandenberg, Ramos, and Altre 1998). In *B. bassiana*, cases of extreme specialisation with isolates being either virulent or avirulent to the tested insect species have only rarely been reported (Riba, Katagiri, and Kawakami 1982; Urtz and Rice 1997). Most studies describe differences among *B. bassiana* isolates in the degree of virulence rather than a distinction into virulence/avirulence. Gradation of virulence of an isolate can however be affected by the bioassay conditions and susceptibility of the insect population of the species being tested. Effects of physical environmental conditions like temperature and moisture on the virulence of isolates have been abundantly documented (e.g. Ferron et al. 1991; Glare and Milner 1991; Vandenberg et al. 1998). With *Nomuraea rileyi*, another entomopathogenic fungus, laboratory bioassay results even with the same isolates under similar conditions in the same lab were reported to vary significantly with bioassays done at different times (Boucias, Stokes, Suazo, and Funderburk 2000). Significant differences in virulence have been observed with *Beauveria brongniartii* isolates tested against two different populations of the same insect species, the European cockchafer *Melolontha melolontha* (Keller, Schweizer, and Shah 1999).

Host specificity in *B. bassiana* was studied taking a sample of isolates from different geographic regions and insect hosts and testing on nine insect species belonging to different taxonomic orders within the class Insecta and two different insect populations within two insect species. It was hypothesised that if the differences in virulence of isolates of *B. bassiana* to different insect species are greater than differences in virulence to different

populations of the same insect species, then, an isolate can be labeled as highly/less virulent against a particular insect species. If the differences in this comparison are not significant, it might be that virulence is determined by the genotype (as regards susceptibility) of the insect population in an insect species rather than the type of insect species *per se*.

Materials and methods

Fungal isolates

The isolates were sampled to constitute representatives from disparate insect hosts collected worldwide from different climatic conditions (Table 1). This sample of 29 *B. bassiana* isolates was DNA fingerprinted by RAPD, AFLP and telomere fingerprinting and found to be very genotypically diverse (Padmavathi 2002; Padmavathi, Uma Devi, Uma Maheswara Rao, and Nageswara Rao Reddy 2003; Uma Devi et al. 2006). The

Table 1. Origin of *Beauveria bassiana* isolates used in the study of insect host specificity.

	Original insect host		
Isolate[a]	Name	Taxonomic order	Geographic origin
ARSEF 326	*Chilo plejadellus*	Lepidoptera	Queensland, Australia
ARSEF 739	*Diabrotica paranoense*	Coleoptera	Goiania, Brazil
ARSEF 1149	*Helicoverpa armigera*	Lepidoptera	Cordoba, Spain
ARSEF 1166	*Helicoverpa armigera*	Lepidoptera	Cordoba, Spain
ARSEF 1169	*Sitona lineatus*	Coleoptera	Senneville, France
ARSEF 1314	*Helicoverpa virescens*	Lepidoptera	La Miniére, France
ARSEF 1315	*Helicoverpa virescens*	Lepidoptera	La Miniére, France
ARSEF 1316	*Helicoverpa virescens*	Lepidoptera	La Miniére, France
ARSEF 1512	*Spodoptera littoralis*	Lepidoptera	La Miniére, France
ARSEF 1788	*Helicoverpa virescens*	Lepidoptera	Spain
ARSEF 2860	*Schizaphis graminum*	Homoptera	Idaho, USA
ARSEF 3041	*Reticulitermus flavipes*	Isoptera	Toronto, Canada
ARSEF 3120	*Senecio* sp.	Homoptera	Yvelines, France
ARSEF 3286	*Spodoptera littoralis*	Lepidoptera	Montpellier, France
ARSEF 3387	*Myzus persicae*	Homoptera	Washington, USA
NRRL 3108	*Ostrinia nubilalis*	Lepidoptera	Unknown
NRRL 20698	*Dysdercus koengii*	Hemiptera	Lima, Peru
NRRL 20699	Unknown	–	Illinois, USA
NRRL 22864	*Glichrochilus quadrisignatus*	Coleoptera	Illinois, USA
NRRL 22865	Unknown	–	Iowa, USA
NRRL 22866	*Pachnaeus litus*	Coleoptera	Florida, USA
ITCC 913	Unknown	–-	The Netherlands
ITCC 1253	*Musca domestica*	Diptera	Mumbai, Central India
ITCC 4521	*Diatraea saccharalis*	Lepidoptera	Karnal, north India
ITCC 4644	*Deanolis albizonalis*	Lepidoptera	Ambajipeta, south India
ITCC 4688	*Helicoverpa armigera*	Lepidoptera	Hyderabad, south India
BB2	*Spodoptera litura*	Lepidoptera	Bangalore, south India
BB3	Soil	–	Bangalore, south India
BB4	*Helicoverpa armigera*	Lepidoptera	Warangal, south India

[a]ARSEF isolates are from USDA-ARS Collection of Entomopathogenic Fungal Cultures, Ithaca, NY; ITCC isolates are from Indian Type Culture Collection, IARI, New Delhi; NRRL isolates are from NRRL culture collection, Peoria, IL; BB isolates are from Indian fields and not yet accessioned.

fungal isolates were cultured on Sabouraud dextrose yeast agar (SDYA) medium in culture tubes placed in an environmental chamber set at $25 \pm 1^{\circ}$C and 90% relative humidity with 16 h L:8 h D cycle. The cultures for every bioassay were established from colonies obtained from streaking conidia stored as glycerol stocks at -20°C. The glycerol stocks were made from conidia harvested from pure cultures of the isolates established from conidia obtained after *in vivo* passage through silkworm (Butt and Goettel 2000). When bioassays are used to compare virulence among isolates obtained from various sources and culture collections whose culture history is seldom known, it is recommended to minimise the variation in virulence due to culture conditions by *in vivo* passage of the isolates (St. Leger, Allee, May, Staples, and Roberts 1991). The conidia of 14-day-old cultures on SDYA slants were harvested by scraping with a sterile steel spatula. For some insects, the conidia were directly used. For others, an aqueous suspension was made with sterile distilled water. Tween 80 (Sigma-Aldrich, USA) was added at a concentration of 0.01% to disperse the conidia in the aqueous medium; the suspension was vortexed. The conidial concentration in the suspension was estimated through hemocytometer counts. The conidial suspension was diluted with 0. 01% Tween till a concentration with countable number of conidia in the hemocytometer was achieved. The conidial counts were made using a light microscope at $400\times$ magnification. The volume of the conidial suspension was made up with 0.01% Tween to the required conidial concentration. For every bioassay, the cultures were established afresh from glycerol stocks. The viability of the conidia used in the treatments was checked as described by Varela and Morales (1996). Data were collected only from such treatments where the conidial viability was $>90\%$. For isolates with lower viability, the bioassay was repeated with conidia from a fresh culture.

Insects

Eight insect species belonging to seven families of five orders were selected for bioassays (Table 2). The lepidopteran insects and mealy bug (*Maconellicoccus hirsutus*) were obtained from second generation laboratory-bred stocks established from field collected insects. The other insects: beetles *Mylabris pustulata* and *Epilachna vigintioctopunctata* and the aphid *Aphis craccivora*, were field-collected. The field-collected insects were kept under observation for a day in the laboratory before treatment. Injured or dead individuals were removed and only active insects were treated. The insects were provided with natural food (Table 2). The insects were placed individually (except *A. craccivora* and *M. hirsutus*) in perforated plastic boxes with lids or plastic dishes whose mouth was closed with a black cloth fastened with a rubber band. Five adult insects of *A. craccivora* and *M. hirsutus* were placed per container. The leaf and flower eating insects were transferred daily to new containers with fresh food. The aphids (*A. craccivora*) were carefully transferred daily to fresh flowers with a fine tip camel hairbrush. Mealy bug (*M. hirsutus*) was left on the same leaf throughout the experiment. A cotton plug was fixed at the tip of the petiole and wetted daily to prevent the leaf from drying and delay leaf senescence (Butt and Goettel 2000). For ants (*O. smaragdina*), diluted (40%, with water) honey was sprayed daily onto a cotton plug in the plastic box in which treated ants were kept.

To compare virulence against different populations within an insect species, two populations from different regions of two insect species, *Spodoptera litura* and *Helicoverpa armigera*, were tested. *H. armigera* collections at both sites were from cotton fields and *S. litura*, from pigeon pea (*Cajanus cajan*) fields. The farmers of the fields were advised to not use chemical insecticides on the crop and the second instar larvae were collected from the field for treatment. For the repeat experiments, the larvae were collected from the same

Table 2. Details of the insects bioassayed with isolates of the entomopathogenic fungus *Beauveria bassiana* to examine host specificity.

		Taxonomic affiliation			Insect treated		
Scientific name	Common name	Order	Family	Stage	Length (mm)	No/replicate	Food
Bombyx mori	Silkworm	Lepidoptera	Bombycidae	2nd instar larva	10–15	10	Mulberry leaves
Spodoptera litura	Army worm (tobacco caterpillar)	Lepidoptera	Noctuidae	2nd instar larva	10–15	10	Castor leaves
Helicoverpa armigera	Boll worm (pod borer)	Lepidoptera	Noctuidae	2nd instar larva	10–15	10	Pigeon pea raw seeds
Chilo partellus	Sorghum shoot borer	Lepidoptera	Pyralidae	2nd instar larva	10–15	10	Sorghum shoot bits
Mylabris pustulata	Blister beetle	Coleoptera	Coccinellidae	Adult	20–25	10	Cajanus flowers
Epilachna vigintioctopunctata	Brinjal beetle	Coleoptera	Coccinellidae	Adult	5–6	10	Brinjal leaves
Aphis craccivora	Pea aphid	Homoptera	Aphididae	Adult	2–2.5	20	Glyricidia flowers
Maconellicoccus hirsutus	Pink hibiscus mealy bug	Hemiptera	Pseudococcidae	Adult	3–4	100	Croton leaves
Oecophylla smaragdina	Red weaver ant (tree nesting ant)	Hymenoptera	Formicidae	Adult worker	8–10	20	Honey

field within a time gap of 4 weeks. The pest density was at its peak during the insect collection time in all the fields from which larvae were collected.

Bioassays

The bioassay with each insect species was set up in a completely randomised block design with appropriate controls (Goettel and Inglis 1997) in an environmental chamber set at $25 \pm 1^{\circ}$C and 90% relative humidity with 16 h L:8 h D cycle. Each treatment was set up as a duplicate and the bioassays with each insect were repeated once. One insect bioassay was done at a time; the bioassays were carried out over a period of 36 months. The description of the insects treated is given in Table 2.

All insect species except *O. smaragdina* and *M. hirsutus* were treated with an aqueous conidial suspension (10^6 conidia/ml) with 0.01% Tween 80 (Sigma-Aldrich). Preliminary bioassays with a few fungal isolates on each insect species that was to be tested were done to decide the conidial concentration suitable to gauge the difference in virulence between the isolates. High conidial doses of *B. bassiana* were found necessary to cause mortality in many insect species; allee effect i.e. requirement of a threshold level of conidia to initiate infection has been observed in bioassays of *B. bassiana* isolates on *Mylabris pustulata* (Uma Devi and Uma Maheswara Rao 2006). Therefore, this conidial concentration was used in the bioassays.

Treatment with aqueous conidial suspension did not elicit high mortality in ants (*O. smaragdina*) and mealy bugs (*M. hirsutus*). Therefore, they were treated with dry conidia harvested from culture slants. The ants were allowed to crawl for 2 min on conidia placed in a Petri dish (Mohan, Aruna Lakshmi, and Uma Devi 1999). Conidia were applied with a fine tip camel hairbrush on mealy bug (*M. hirsutus*). The number of conidia that attached to an insect in this mode of treatment was estimated as described by Mohan et al. (1999). Briefly, the treated insect was dipped in water (volume enough to drown the insect), Tween at a concentration of 0.01% was added and vortexed thoroughly. The insect was removed and the spore count in the water was made through haemocytometer counts. An inoculum concentration of $\sim 10^9$ conidia/100 insects could be dispensed on mealy bugs and $\sim 10^7$ conidia/insect for ants through the methods of application described. The controls were treated with heat (100°C) killed conidia.

In treatments with aqueous conidial suspension, for each insect species, the volume sufficient to drench the insect was determined and equal volume was dispensed on all insects with a Gilson® micropipette. As the aphids were very small, 20 were placed on a flower bud and the inoculum was dispensed onto them. The concentration of the conidial suspension was adjusted such that each volume aliquot dispensed per 20 insects (aphids) contained $\sim 2 \times 10^6$ conidia. A volume of 100 µL was required to drench the larvae of *B. mori*, *S. litura*, *H. armigera* and *C. partellus*, 150 µL for *M. pustulata* and 40 µL for *E. vigintioctopunctata*. The controls were treated with an equal volume of water with 0.01% Tween.

Mortality of the insects was recorded daily post treatment in all insect bioassays except with *C. partellus* (sorghum shoot borer). In bioassays with *C. partellus*, mortality was recorded at 4-day intervals: the larvae tunneled into shoots provided as diet; to minimise injury to the larvae while tearing open the shoots, the shoots were cut open at 4-day intervals to observe the larvae.

Treated dead insects were placed singly in humid chambers (Petri dish lined with moist blotting paper) to facilitate mycosis. The data were recorded in larval bioassays until all the larvae in the controls pupated, and in bioassays on adult insects, the day from which no further mortality was observed in the treated insects. The duration of the bioassay was 7

days for *A. craccivora*, *O. smaragdina* and *M. hirsutus*, 8 days for *H. armigera* and *S. litura*, 14 days for *B. mori* and *M. pustulata*, 16 days for *E. vigintioctopunctata* and 30 days for *Chilo partellus*.

To examine if differences in virulence exist based on difference in the genotype of insects within an insect species, bioassays were done with a subset (five isolates) of *B. bassiana* sample on two populations of *Helicoverpa armigera* and *Spodoptera litura* collected from different locations. Two isolates which caused high mortality on all the tested eight insect species were included in this sample. The two populations of each insect species were tested simultaneously with the same conidial inoculum of the *B. bassiana* isolate. The experiment with the two populations was set up as a completely randomised block design with three replicates for each treatment. The experiment was repeated with the second set of field collected larvae with inoculum from fresh cultures of the *B. bassiana* isolates.

Data analysis

In each treatment, the cumulative mortality on the last day of observation was corrected for control mortality (Abbott 1925) and percent $\sqrt{}$arcsine transformed to normalise data and back transformed (Gomez and Gomez 1984). The number of insects with mycosis (cadavers with external growth of fungus) was computed as percent proportion of the dead insects. The mean $\pm$SE of percent mortality and percent mycosis of the two replicates of each isolate was calculated. The mean values were back transformed to normalise the data (Gomez and Gomez 1984). Median lethal time was calculated from the cumulative mortality data using survival analysis with Weibull distribution (Lee 1992).

The results of bioassays are summarised in Tables 3 and 4. The mortality and mycosis values are rounded to the nearest whole number. Standard errors (SE) in no instance were so high as to indicate a significant difference between replicate experiments; they are not mentioned to simplify the table. The results of bioassays with entomopathogenic fungi conducted at different times in the same laboratory and with the same isolates on the same insect in different laboratories are reported to vary (Boucias et al. 2000). Therefore, less importance was given to the absolute values. The isolates were graded broadly into three classes – high, medium and low (A, B and C, respectively) (Yip, Rath, and Koen 1992) for the three virulence characteristics (mortality, mycosis and median lethal time). For mortality and mycosis, taking the range as 100 (0–100%), 0–33% was taken as low (C), 34–67% as medium (B) and 68–100% as high (C). For values in the boundaries of the grades, the SE value was added to the mean for grading. For classifying the speed of kill, a day was subtracted from the total time duration of the bioassay (because mortality observations were started 1 day after treatment) and divided by three to set the upper boundary for median lethal time for each of the three (A, B, C) classes. The statistical analysis was done using SPSS 7.5 for windows (SPSS Inc. 1989–1996).

Results

Virulence comparison of isolates to different insect species

Mortality

All the *B. bassiana* isolates in the sample were found pathogenic to all the insect species tested (Table 3). A majority of the isolates caused high ($>$67%) mortality in all tested insects except *M. hirsutus* and *C. partellus*; 28 isolates on *A. craccivora*, 27 on *O. smaragdina* and at least 25 of the 29 *B. bassiana* isolates tested, on the other insect

Table 3. Mortality, mycosis and median lethal time (LT_{50}) computed from the data of the laboratory bioassays of a worldwide sample of *B. bassiana* on eight insect species.

	% Mortality[a]								% Mycosis[a]								LT_{50} (days)[b]							
Isolate	Bm	Sl	Cp	Ev	Mp	Ac	Mh	Os	Bm	Sl	Cp	Ev	Mp	Ac	Mh	Os	Bm	Sl	Cp	Ev	Mp	Ac	Mh	Os
ARSEF 326	59	**100**	50	60	**99**	**98**	**72**	57	0	0	50	**100**	40	15	57	**74**	7.5	**1.7**	14.4	7.0	8.9	3.7	4.9	6.2
ARSEF 739	**100**	**100**	**100**	50	**98**	**99**	53	**100**	67	30	**80**	**100**	44	0	56	**98**	5.3	**2.7**	6.1	7.8	**2.7**	3.4	6.4	4.1
ARSEF 1149	**100**	**100**	60	**100**	**85**	**99**	65	**100**	**80**	**80**	**84**	**88**	**100**	**68**	**69**	**100**	4.1	**2.2**	13.9	5.6	5.1	3.6	4.7	**3.9**
ARSEF 1166	**100**	**80**	**70**	**100**	**100**	**73**	51	**69**	20	50	**78**	38	20	24	52	**65**	**3.9**	**3.3**	11.9	11.0	**2.9**	4.3	6.2	4.9
ARSEF 1169	**100**	**81**	40	**70**	**75**	**100**	45	**96**	0	8	50	57	49	0	**92**	**94**	5.5	**3.3**	E	8.3	7.9	4.4	E	4.3
ARSEF 1314	**100**	**80**	40	**90**	59	**100**	55	**100**	50	37	**79**	**70**	49	6	49	**100**	4.4	**1.2**	E	5.4	5.6	6.1	5.4	**3.9**
ARSEF 1315	40	60	30	**80**	56	**100**	62	**90**	0	32	**78**	**93**	42	27	51	**100**	10.1	5.3	E	8.3	5.3	3.8	5.4	**3.9**
ARSEF 1316	**80**	**93**	50	**90**	**100**	**100**	**71**	**100**	0	33	**85**	19	33	**72**	51	**100**	7.7	**3.6**	14.9	5.4	**2.4**	3.5	5.4	4.3
ARSEF 1512	**100**	**100**	50	**81**	**99**	**100**	48	**99**	30	**100**	41	**78**	63	25	58	**87**	4.4	**2.3**	14.4	6.7	7.3	3.4	7.1	4.2
ARSEF 1788	**100**	**100**	40	**100**	**100**	**82**	50	**83**	**90**	**100**	**79**	**72**	33	23	42	**93**	**3.7**	**3.2**	E	5.1	**3.4**	5.6	6.1	5.1
ARSEF 2860	**100**	**81**	40	**90**	**98**	**99**	**90**	**100**	**100**	**100**	56	66	**70**	20	**75**	**100**	5.2	4.1	E	8.7	**3.2**	3.5	4.1	4.2
ARSEF 3041	**70**	**81**	57	**100**	**98**	**100**	41	**90**	0	0	**68**	**75**	**89**	27	51	62	7.9	4.9	13.9	5.6	6.7	3.9	E	4.5
ARSEF 3120	**100**	50	40	**70**	**100**	65	21	**98**	20	20	**94**	20	13	0	**83**	**100**	5.1	6.3	E	6.8	5.6	7.5	E	**3.5**
ARSEF 3286	**80**	**70**	40	**80**	**85**	**83**	**70**	**87**	0	28	50	**68**	16	0	32	**78**	6.5	4.2	E	6.4	11.6	7.0	3.9	**3.8**
ARSEF 3387	**93**	**100**	60	60	**99**	**79**	45	**84**	22	0	**68**	**87**	0	0	27	60	6.4	**3.7**	14.6	11.2	8.6	6.1	E	4.6
NRRL 3108	**100**	60	**70**	**100**	**100**	**99**	70	**100**	60	**100**	**80**	29	60	33	**77**	**100**	5.0	5.0	13.4	5.6	**3.5**	4.3	5.5	**3.3**
NRRL 20698	**93**	**70**	40	**100**	**93**	**100**	42	**90**	50	**70**	50	38	0	38	**75**	**100**	4.4	4.3	E	6.7	6.3	**2.9**	E	4.4

Table 3 (*Continued*)

	% Mortality[a]								% Mycosis[a]								LT_{50} (days)[b]							
Isolate	Bm	Sl	Cp	Ev	Mp	Ac	Mh	Os	Bm	Sl	Cp	Ev	Mp	Ac	Mh	Os	Bm	Sl	Cp	Ev	Mp	Ac	Mh	Os
NRRL 20699	**70**	**81**	50	**80**	**99**	**100**	**83**	**97**	10	**91**	38	**78**	**85**	20	57	**100**	8.8	4.1	15.5	6.4	4.2	3.5	5.5	**2.9**
NRRL 22864	**100**	**100**	**100**	**100**	**99**	**99**	61	**100**	**90**	**100**	**77**	**76**	50	**77**	**71**	**100**	**2.1**	**1.8**	10.0	6.6	**3.2**	3.4	5.1	4.3
NRRL 22865	**70**	**90**	50	**80**	**85**	**99**	35	**75**	10	33	47	12	0	0	**76**	**100**	7.9	**3.3**	14.9	5.9	11.1	**2.4**	E	5..2
NRRL 22866	**100**	**80**	50	**80**	63	**97**	38	66	20	25	**73**	**96**	2 5	12	**82**	62	6.2	4.3	14.8	5.0	3.8	3.7	E	5.8
ITCC 913	**100**	**100**	60	**100**	**75**	**99**	**82**	**97**	50	11	**81**	14	66	40	**82**	**97**	5.0	**0.7**	13.7	**2.3**	3.8	3.2	3.7	4.1
ITCC 1253	**90**	**100**	50	**100**	**100**	**89**	40	**96**	7	**70**	33	**72**	40	56	**78**	**91**		6.3	**2.1**	14.9	7.2	4.8	E	4.4
ITCC 4521	**100**	**93**	50	**80**	56	**94**	**68**	**100**	0	11	60	**87**	30	9	**78**	**81**	4.4	4. 9	14.4	6.2	7.8	4.3	4.6	**3.0**
ITCC 4644	47	**100**	50	**70**	**75**	**92**	**68**	**100**	0	10	**95**	26	50	**80**	**77**	**99**	9.0	**2.3**	14.6	8.8	5.6	4.4	4.7	**3.2**
ITCC 4688	**100**	**100**	**80**	**90**	**88**	**92**	58	**68**	40	40	48	**100**	0	0	36	65	5.0	**2.9**	8.9	7.1	8.4	**1.6**	4.7	5.6
BB2	**100**	**100**	**80**	**100**	**100**	**100**	**90**	**100**	**80**	30	**94**	14	**75**	**75**	**98**	**100**	4.2	**3.8**	8.1	5.0	5.5	**3.5**	**2.8**	**3.5**
BB3	**100**	**81**	**70**	**70**	**100**	**92**	**72**	**96**	**80**	**75**	47	**100**	58	**82**	**99**	**100**	**3.7**	**3.8**	9..2	5.2	7.3	**3.0**	4.0	4.3
BB4	**100**	**81**	**90**	**100**	**100**	**100**	37	**71**	20	50	**94**	13	**86**	**67**	**93**	**89**	5.7	**2.9**	10.5	3.5	8.8	3.3	E	5.0

Numbers in bold indicate code A, in regular, B and underlined, C; codes A, B and C stand for high, medium and low virulent; for mortality and mycosis A, >67%; B, 33–67%; C, <33% (the range 100 (0–100) is divided by 3 for setting the boundaries for each class); for LT_{50}, for Bm, Sl and Os: A, ≤4; B, 4.1–7; C, >7; for Ac and Mh: A, ≤3; B, 3.3–9.7 ; C, >9.7 and for Cp, Mp and Ev: A, ≤5.3; B, 5.4–9.7; C, >9.7 (from the time duration of the bioassay, one day was subtracted and divided by 3 to set the boundaries for each class). Bm, *Bombyx mori*; Sl, *Spodoptera litura*; Cp, *Chilo partellus*; Ev, *Epilachna vigintioctopunctata*; Mp, *Mylabris pustulata*; Ac, *Aphis craccivora*; Mh, *Maconellicoccus hirsutus* and Os, *Oecophylla smaragdina*. [a]Mortality data are Abbott corrected for control mortality (Abbott 1925). The % mortality in controls in the two repeat experiments are for Bm, 0/10; Sl, 0/0; Cp, 15/0; Ev, 20/10; Mp, 10/10; Ac, 20/0; Mh, 15/8; and Os, 10/0 (values in the two replicate experiments given on either side of /). The values represent mean of two bioassays with each treatment set up in duplicate, arc sine transformed and rounded up to nearest whole number. [b]LT_{50} (median lethal time) computed from survival analysis table using Weibull distribution; E stands for error, median lethal time could not be computed because total mortality caused is less than 50%.

Table 4. Range of the three virulence characteristics: mortality, mycosis and median lethal time and number of isolates in each virulence class[a] as observed in the laboratory bioassays with a worldwide sample of *Beauveria bassiana* on eight insect species.

	% Mortality[b]				% Mycosis[b]				LT_{50}(days)[c]			
		No of isolates coded				No of isolates coded				No of isolates coded		
Insect	Range	A	B	C	Range	A	B	C	Range	A	B	C
Bombyx mori	40–100	26	3	0	0–100	6	6	17	2.1–10.1	4	18	7
Spodoptera litura	50–100	26	3	0	0–100	10	4	15	0.7–6.3	19	10	0
Chilo partellus	30–100	8	20	1	37–98	17	12	0	6.1–14.9	0	4	25
Epilachna vigintioctopunctata	50–100	26	3	0	12–100	17	4	8	2.4–11.6	1	26	2
Mylabris pustulata	56–100	25	4	0	0–100	6	14	9	2.4–11.6	7	19	3
Aphis craccivora	65–100	28	1	0	0–82	7	5	17	1.6–7.5	5	24	0
Maconellicoccus hirsutus	21–90	10	18	1	27–99	16	11	2	2.8–7.1	1	9	19
Oecophylla smaragdina	57–100	27	2	0	60–100	25	4	0	2.9–6.2	10	19	0

[a]Classes coded as A, B and C for high, medium and low virulence. [b]A, >67%; B, 34–66%; and C, <=33. [c]One day was subtracted from the total duration of the bioassay and divided by 3 to set the boundaries for each class

species (Tables 3 and 4). With *C. partellus*, only eight isolates caused high mortality and with *M. hirsutus*, 10 isolates (Tables 3 and 4). Most of the isolates caused medium mortality on these two insects (Tables 3 and 4). The percent mortality in the controls of each insect bioassay are given in Table 3. In all of the insect bioassays, including those done with field collected samples (*M. pustulata*, *O. smaragdiana*, *A. craccivora*), the few insects that died among the controls showed no sign of fungal infection.

Mycosis

Only one isolate, ARSEF 1149, graded high in mycotic potential on all insect species (Table 3). With others, the mycotic potential of an insect was not uniform on different insect species (Table 3). Some of the isolates caused no mycosis on some insect species (Table 3).

Median lethal time

When the speed of killing of an isolate across all the eight insects was compared, no isolate showed a uniform behaviour (Table 3). The isolates could not be rated as fast/slow killers; the LT_{50} values of an isolate varied with different insect species.

Virulence comparison between two populations of two insect species

Great differences were observed in the virulence comparison of isolates to different populations of *S. litura* and *H. armigera*, The isolates caused high mortality (grade A) in one population but low mortality (grade C) on another, the difference being highly significant (Table 5). Among the isolates of this subset sample were isolates BB3 and BB4 which caused high mortality (graded A) on all the eight insect species tested in this study (Table 3). With respect to mycotic potential also, the difference of an isolate to different

populations of an insect species was as variable (Table 5) as the differences observed with different insect species (Table 3). Just as with the other two virulence characters, the difference in speed of kill of an isolate to different insect populations of an insect species was also very drastic; the LT_{50} values could not be computed for an isolate with one population due to less than 50% mortality caused while with another population, the LT_{50} values ranged between 3.89–4.65 days in *H. armigera* and 2.26–3.84 days in *S. litura* (Table 5). Thus, the difference in virulence of an isolate to two insect populations within an insect species was even more than the differences observed between isolates with different insect species. More than three-fourths of the *B. bassiana* isolates in the sample caused high mortality in most of the insect species (Tables 3 and 4).

Discussion

All the 29 *B. bassiana* isolates bioassayed were found pathogenic to all the eight insect species tested. While considering host specificity, among the three virulence parameters of the fungal isolate, mortality, mycosis and median lethal time, mortality could be taken as the main trait. A majority (at least 25 of the 29 tested) of the *B. bassiana* isolates were found highly virulent to six of the eight insect species tested. Fewer isolates were highly virulent to *C. partellus* and *M. hirsutus*. In their studies on host specificity in *B. bassiana*, Viaud et al. (1996) and Maurer et al. (1997) concluded that specificity might be a characteristic of the insect species rather than the fungal isolate. In the present study, significant differences were observed in mortality caused by an isolate on different populations within *H. armigera* and *S. litura*. Therefore, it is not possible to conclude if these two insect species (*C. partellus* and *M. hirsutus*) are in general less susceptible to *B. bassiana*, or if the tested insect population of these two insects was genotypically less susceptible to fungal infection. Significant differences in susceptibility between different insect populations of an insect species to fungal pathogens have been reported (Milner 1985; Stephen and Fichter 1990; Keller et al. 1999). The tunnelling behaviour of *C. partellus* and the waxy coat of *M. hirsutus* may have affected the adhesion of conidia, essential to initiate infection resulting in less mortality in these insect species.

The laboratory bioassays described here were not conducted to evaluate the dose response to arrive at an accurate estimate of virulence of an isolate given the sample size of *B. bassiana* and the number of insect species tested. Thus, they are preliminary. However, the results clearly point to the trends. A strict host specialisation with an isolate being highly virulent to one or a group of insect species with very low or no virulence to another group of insect species is not evident in the *B. bassiana* sample in the present study. Successful pathogenesis was found to be affected as much by the genotype (with respect to susceptibility) of the insect population as the type of insect species. Humber (1991) hypothesized that the entomogenous habit of *B. bassiana* may be relatively new and therefore it is poorly adjusted to the host. *B. bassiana* is described as a hemi-biotroph (Butt and Goettel 2000) and is not fastidious of diet.

The *B. bassiana* sample examined in the present study is constituted by isolates collected worldwide from disparate insect hosts with immense genotypic variability (Padmavathi 2002; Padmavathi et al. 2003; Uma Devi et al. 2006). Therefore, it can be taken as representative of the large spectrum of diversity in this species complex including at least, some of the specialised physiological races or sub-species. Fargues, Duriez, Popeye, Robert, and Biguet (1981) observed that host specialisation can serve as a taxonomic criterion in obligate parasites, but, in entomopathogenic hyphomycetous fungi like *B. bassiana*, because of their facultative saprotrophic habit, it may not be feasible to use this criterion to

Table 5. Comparison of the virulence of five representative isolates of *Beauveria bassiana* sample in bioassays (at 25°C) on two different populations of *Helicoverpa armigera* and *Spodoptera litura.*

	Helicoverpa armigera					*Spodoptera litura*				
Isolate	Pop[a]	Mortality (%)[b]	Mortality range[c]	Mycosis (%)[b]	LT_{50} (days)[d]	Pop[a]	Mortality (%)[b]	Mortality range[c]	Mycosis (%)[b]	LT_{50} (days)[d]
ITCC 4644	1	30 (0) C	55	7 C	E	1	20 (0) C	80	13 C	E
	2	85 (10) A		65 A	4.69	2	100 (10) A		10 C	2.26
ITCC 4688	1	35 (0) C	55	2 C	E	1	55 (0) B	45	3 C	6.72
	2	90 (10)A		81 A	3.69	2	100 (10) A		40 B	2.29
BB2	1	20 (0) C	72	0 C	E	1	30 (0) C	70	20 C	E
	2	92 (0) A		38 B	4.05	2	100 (20) A		31 C	3.84
BB3	1	12 (10) C	62	2 C	E	1	30 (0) C	53	0 C	E
	2	74 (20) A		85 A	4.12	2	83 (0) A		75 A	3.81
BB4	1	12 (0) C	84	0 C	E	1	38 (10) B	45	3 C	E
	2	96 (0) A		80 A	3.89	2	83 (0) C		50 B	2.94

[a]Populations from two different loctions: population 1 from suburbs of Hyderabad (AP, India), population 2 of *H. armigera* is from Kavuru village in Guntur district (AP, India) ~200 miles from Hyderabad and of *S. litura* is from Bangalore (Karnataka, India) ~500 miles from Hyderabad. [b]Values are Abbott corrected for control mortality (Abbott 1925). Values in brackets represent % mortality in controls. The values are mean of two experiments each set up in duplicate $\sqrt{\ }$ arc sine transformed and rounded to the nearest whole number. A, B, C represent the grades: A, >67%; B, 33-67%; C, <33%. The observed differences in mortality between the two populations were found highly significant in ANOVA test (SPSS Inc 1996). ANOVA values for difference between the two populations of *Helicoverpa armigera* are df = 3, $F = 10.84$, $P = 0.0012$ and for *Spodoptera litura* are df = 4, $F = 112.99$, $P = 0$. [c]Range between the two populations. [d]LT_{50} (median lethal time) computed from survival analysis table using Weibull distribution; E stands for error – median lethal time could not be computed because total mortality caused is less than 50%.

differentiate the different 'species' in the species complex. A similar conclusion was arrived in the study of host range of *Aspergillus flavus*, an ascomycetous fungus with highly polymorphic and complex populations (St. Leger, Screen, and Shams Pirzadeh 2000). Most of the *Aspergillus flavus* strains cause disease in both plants and animals; the same *A. flavus* strain infects diverse lepidopteran insect species (St. Leger et al. 2000).

Beauveria bassiana has thus a generalist nature. From the study of *Metarhizium anisopliae*, another entomopathogenic mitosporic fungus similar to *B. bassiana* in having a very wide host range and a saprophytic existence in soil, Bidochka, Kamp, Lavender, Dekoning, and De Croos (2001) concluded that 'the search for highly virulent isolates directed at certain insect pests may be inherently flawed, since virulent isolates have comparable facility to infect susceptible insects but have large discrepancies in their abilities to tolerate certain environmental conditions'. They recommend that habitat preferences rather than virulence should be considered as a feature for selecting fungal strains to be used in biocontrol efforts. Carruthers and Soper (1987) speculated that the pathogen (fungal) may be limited in the field by environmental conditions and/or spatial and temporal interactions with its host rather than the lack of pathogenecity.

With the generalist character of *B. bassiana*, it is not necessary to choose isolates suitable for each one of the multitude insect pests, rather isolates suitable for different habitats (environmental conditions). An isolate suitable for a particular habitat would be sufficient to deal with most of the insect pests in that area, just like the nonspecific chemical pesticides. A broad host spectrum biopesticide based on *B. bassiana* may, however, may have the risk of causing undesirable effects on non-target beneficial organisms like chemical insecticides. Goettel and Hajek (2001) observe that too much concern over effects of entomopathogenic fungi on non-target organisms is not warranted. The physiological host range as evident from laboratory bioassays may not be similar to ecological host range and a mycopesticide formulation targeted against a dense pest population may not have disastrous effects on the non-target organisms.

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HETEROGENEITY OF TWO *BEAUVERIA BASSIANA* STRAINS REVEALED BY BIOCHEMICAL TESTS, PROTEIN PROFILES AND BIO-ASSAYS ON *LEPTINOTARSA DECEMLINEATA (COL. : CHRYSOMELIDAE)* AND *COLEOMEGILLA MACULATA LENGI (COL. : COCCINELLIDAE)* LARVAE

S. I. TODOROVA (1), J.-C. CÔTÉ (2), P. MARTEL (2), & D. CODERRE (1)

(1) Département des Sciences Biologiques, Université du Québec à Montréal.
C.P. 8888, Succ. A, Montréal, Canada H3C 3P8
(2) Station de Recherches, Agriculture Canada, 430 Boul. Gouin, St-Jean-sur-Richelieu, Québec, Canada J3B 3E6

Biochemical profiles on API Rapid CH* strips and protein profiles on polyacrylamide gels in the presence of sodium dodecyl sulfate were used to distinguish two strains of the entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin, ARSEF 2991 and ATCC 44860. Next, the toxicity of these two strains was determined at concentrations of 10^2, 10^4, 10^6 and 10^8 blastospores/ml on larvae of the Colorado potato beetle *Leptinotarsa decemlineata* Say (Coleoptera : Chrysomelidae) and of its predator, the spotted ladybird beetle, *Coleomegilla maculata lengi* Timberlake (Coleoptera : Coccinellidae).

Both strains were highly toxic to *L. decemlineata* larvae. However, the two strains exhibited different levels of toxicity for *C. maculata* larvae : ARSEF 2991 was toxic, whereas ATCC 44860 caused little coccinellid larval mortality.

KEY-WORDS : Entomopathogenic fungus, strain, blastospores, larval mortality, *Coleomegilla maculata lengi, Leptinotarsa decemlineata.*

Among fungi, *Beauveria bassiana* (Balsamo) Vuillemin is one of the most widespread entomopathogen (Roberts & Yendol, 1971 ; Tanada & Kaya, 1993). Many different *B. bassiana* strains exist, some of these are toxic, at varying degrees, to the Colorado potato beetle *Leptinotarsa decemlineata* Say (Ferron, 1981 ; Anderson *et al.*, 1989).

B. bassiana has been experimentally tested for the control of *L. decemlineata* in ex-USSR, in Poland (Bajan *et al.*, 1987), in ex-Czecoslovakia (Weiser, 1987), in France (Fargues *et al.*, 1980, 1991) and in USA (Hajek *et al*, 1987 ; Groden & Lockwood, 1991). A commercial formulation made from conidiospores has been available since 1977 in Ukraine under the name Boverin™. The dose recommended by the manufacturer is $6x10^9$ conidiospores/g (Lippa, 1985).

In Quebec, Canada, the Colorado potato beetle, *Leptinotarsa decemlineata* Say (Coleoptera : Chrysomelidae), is the insect pest against which the greatest quantity of chemical insecticides is used (Chagnon *et al.* 1990). This insect, however, has rapidly developed a resistance to a great variety of insecticides (Martel, 1987) which has led to an increase in the dosage used in the field. In addition to the many negative consequences on the environment, insecticides seriously reduce populations of the Colorado potato beetle's natural enemies.

One of these natural enemies, the spotted ladybird beetle, *Coleomegilla maculata lengi* Timberlake (Coleoptera : Coccinellidae), should be regarded as a very important component in an integrated pest management system in Canada (Boiteau, 1983). It is a polyphagous predator that can significantly reduce Colorado potato beetle populations (Groden *et al.*, 1990). Each *C. maculata* adult can attack 20 eggs in 48 h (Hazzard & Ferro, 1991) and consume, on average, 11.2 young *L. decemlineata* larvae (Groden *et al.*, 1990).

Few studies have been conducted to study the impact of entomopathogenic microorganisms on *C. maculata*. In a laboratory study, Giroux *et al.* (1993) have shown that *C. maculata* larvae and adults are not sensitive to M.One™, a commercial *Bacillus thuringiensis* var. *san diego* preparation, even at doses 10 times higher ($5.6x10^8$ Colorado Potato Beetle International Units (CPBIU)/l) than recommended by the manufacturer. Fungi, however, appear to be less selective than bacteria. A study of the activity of *B. bassiana* strain ARSEF 731 has shown that the $7.2x10^4$ conidiospores/mm^2 dose was not toxic to adults of *C. maculata* after contamination by ingestion, while this strain caused 60 % mortality following treatment by contact (Lord *et al.*, 1988). According to Goettel *et al.* (In Lord *et al.*, 1988), 16 coccinellid species can be infected by *B. bassiana*. Iperti (1966) has frequently observed dead *Semiadalia undecimnotata* Schneider (Coleoptera : Coccinellidae) adults infected by *B. bassiana* at hibernation sites, especially at altitudes of less than 1000 m.

The USDA-ARS Collection of Entomopathogenic Fungal Cultures (Humber, 1992) contains close to 1000 different *B. bassiana* isolates, some of which isolated from *L. decemlineata*, others from Coccinellidae. The study of the heterogeneity of the species either at the biochemical level or at the level of host specificity has received little attention. Clearly, some methods were needed to distinguish rapidly between *B. bassiana* isolates and to get a picture of the heterogeneity of the species. The aim of the current study was to determine first, whether two strains of *B. bassiana* could be distinguished using biochemical methods or protein profiles on SDS-PAGE and second, whether different strains of *B. bassiana* could exhibit different host toxicity against *C. maculata* and *L. decemlineata* larvae.

API Rapid CH* strips contain various carbon sources and are used to identify biochemical or nutritional characteristics of microorganisms (Anonymous, 1988). API Rapid CH* profiles are routinely used to distinguish between bacteria at the genus, species, variety and isolate level (Logan & Berkeley, 1984). API Rapid CH* strips were tested in this study for their ability to discriminate rapidly between fungal isolates. Protein profiles generated by electrophoresis on polyacrylamide gels in presence of sodium dodecyl sulfate have been used extensively to, among other things, discriminate between microorganisms. They were used here to determine whether they were sensitive enough to distinguish between two *B. bassiana* isolates.

We chose to work with blastospores because they usually germinate within 48 h following infection, compared with three to four weeks for conidiospores (Müller-Kögler & Samsinakova, 1969). *B. bassiana* strain ARSEF 2991 was chosen because it is indigenous to Quebec, Canada, and was originally isolated from *L. decemlineata*, the target insect to be controlled. The other strain chosen needed to be as different as possible from ARSEF 2991 in order to test the discriminatory level of API Rapid CH* test, protein profiles, and toxicity level on *C. maculata* and *L. decemlineata* larvae. ATCC 44860 appeared as a good candidate because it had been isolated from soil in Georgia, U.S.A.

MATERIALS AND METHODS

STRAINS

Two *Beauveria bassiana* strains were used. The ARSEF 2991 strain was obtained from T. Searle (Macdonald College, McGill University, Montreal, Canada). It was initially isolated from dead adult *L. decemlineata*, collected in the summer of 1988 in a potato field near Ste-Clothilde (Québec, Canada). The strain is registered at the USDA-ARS collection in Ithaca, New York (Humber, 1992). The ATCC 44860 strain was purchased from the American Type Culture Collection in Rockville, Maryland. It was initially isolated from a soil sample in Georgia, USA (Hammill, 1970).

BLASTOSPORE PRODUCTION

B. bassiana blastospores were used to contaminate spotted ladybird beetle larvae. Blastospores were produced in 300 ml of sporulation medium for three days, incubated at 25 °C with agitation, as described by Alioshina *et al.* (In Ferron, 1981) with some modifications. The pH was adjusted to 5 and sucrose was replaced with sorbitol to increase blastospore production (Samsinakova *et al.*, 1981). The culture was centrifuged at 8000 RPM in a Beckman T-865 rotor for 10 min at 4 °C in a Beckman J2-21 M high speed centrifuge. The supernatant was discarded and the pellet resuspended in 50 ml of 0.85 % NaCl. The blastospores were observed under a microscope and the titer determined by serial dilutions.

BIOCHEMICAL PROFILES

Blastospores from the *B. bassiana* strains were transferred on 2YT agar plates (10 g Yeast extract, 16 g Bacto-tryptone, 5 g NaCl, 15 g Agar per litre) and incubated at 30 °C for 48 h. The cultures were resuspended in 3 ml 0.85 % NaCl. The biochemical profiles were determined on API Rapid CH* strips following the manufacturer's recommendations (Anonymous, 1988).

PROTEIN PROFILES

Total proteins were separated by SDS-PAGE electrophoresis (Laemmli, 1970). The gel (20 cm x 20 cm x 1.5 mm) contained 10 % acrylamide for separation and 4 % acrylamide for protein concentration. The electrophoresis was carried out under continuous current at 60 mA for 5 h. The proteins were stained with Coomassie Blue R-250 (methanol : acetic acid : water, 50 :10 :40) for 1 h, then unstained with a mixture of methanol : acetic acid : water (7 :5 :88).

REARING OF COCCINELLIDS

Adults *C. maculata* were collected in the spring of 1992 at hibernation sites situated in the vicinity of corn fields in St-Hyacinthe, Quebec, Canada (45°39'N, 72°56'W). They were placed in cages with a diet designed to stimulate egg laying. The diet comprised pollen, aphids *Aphis fabae* (Scop.) and *Anagasta kuehniella* (Zeller) eggs. To avoid cannibalism, each larva, upon hatching, was individually reared in Petri dishes on a pollen diet. The conditions were kept constant at 25 °C, 70 % humidity and a 16L :8D photoperiod.

REARING OF COLORADO POTATO BEETLES

L. decemlineata larvae were obtained from a mass rearing maintained from individuals collected in June 1990 in Trois-Pistoles, Quebec, Canada (48°07'N,69°10'W). Rearing was done on potato plants of the "Kennebec" variety under controlled conditions (23 °C, 40 % RH, 16L :8D).

BIOASSAYS

Blastospore concentrations of 10^2, 10^4, 10^6 and 10^8 colony forming units (CFU)/ml of each strain were used for bioassays. The two strains were diluted in 0.85 % NaCl and Triton X-100 was added to the solution at a final concentration of 0.1 %.

The contaminated pollen, an easily accessible and inert food, was offered as a diet to first instar *C. maculata* larvae. A 25 g quantity of wildflower pollen was mixed with 25 ml of the blastospore solution. After a vacuum filtration, the pollen was dried at 60 °C for 3 h. This procedure was repeated for all concentrations. To observe the toxicity of the *B. bassiana* samples on *L. decemlineata*, the larvae were transferred to Petri dishes. A Whatman 3M filter paper moistened with 0.5 ml distilled water was placed at the bottom of each Petri dish. Potato leaves were sprayed with 1 ml of each *B. bassiana* concentration, then dried and placed on the filter paper. In each Petri dish, 30 first or second instar larvae were placed on a contaminated leaf which was replaced daily. Two Petri dishes were used for each blastospore concentration.

Mortality was noted for each blastospore concentration every 24 h over a ten day period. Results were analysed with a χ^2 test (Statview, version 1.03, Macintosh®) (Abacus Concepts Inc., 1988). The percentage mortality was corrected following Abbott's (1925) method.

RESULTS

COMPARISON OF THE TWO *B. BASSIANA* STRAINS

After a three day incubation period in the sporulation medium at 25 °C, sporulation of the two *B. bassiana* strains occurred. Blastospores from both strains appear indistinguishable from each other under the microscope (800X) based on size and shape or from other *B. bassiana* blastospores as reported in the literature (Weiser, 1972).

However, the API Rapid CH* profiles of the two strains are different (table 1). The ARSEF 2991 strain is able to acidify the following components. L-arabinose, galactose, sorbose, mannitol, sorbitol, salicin, melibiose, melezitose, gentiobiose and D-turanose. Strain ATCC 44860, however, shows a negative reaction for all these tests. It differs in the lack of acidification of erythritol, adonitol, α-methyl-D-glucoside and lactose, while the ARSEF 2991 strain possesses slight activity. Both strains acidify ribose, arbutin, and cellobiose, yet strain ARSEF 2991 is more active in this respect.

Protein electrophoretic analysis indicates significant differences between both strains (fig. 1). Strain ARSEF 2991 possesses a predominant protein band with a low molecular weight, estimated at 34 kDa, while strain ATCC 44860 differs in the presence of a protein of 70 kDa absent in strain ARSEF 2991.

TOXICITY OF THE TWO *B. BASSIANA* STRAINS ON *L. DECEMLINEATA* LARVAE

After 10 days, the two strains resulted in mortality significantly different to the control at the following concentrations: 10^4, 10^6 and 10^8 blastospores (blsp)/ml (χ^2 ; df = 3 ;

TABLE I

Carbon sources utilisation profiles of B. bassiana *strains determined on API Rapid CH* strips*

	CTRL	GLY	ERY	DARA	LARA	RIB	DXYL	LXYL	ADO	MDX	GAL	GLU	FRU	MNE	SBE	RHA	DUL	INO	MAN	SOR	MDM	MDG	NAG	AMY	ARB
ATCC 44860	0	1	0	0	0	3	0	0	0	0	0	5	5	5	0	0	0	0	0	0	0	0	5	0	3
ARSEF 2991	0	1	1	0	5	5	1	0	1	0	5	5	5	5	5	0	0	0	5	5	0	1	5	0	5

	ESC	SAL	CEL	MAL	LAC	MEL	SAC	TRE	INU	MLZ	RAF	STA	GLG	XLT	GEN	TUR	LYX	TAG	DFUC	LFUC	DARL	LARL	GNT	2KG	5KG
ATCC 44860	5	0	3	5	0	0	5	5	0	0	0	5	5	0	0	0	0	0	0	0	0	0	0	0	0
ARSEF 2991	5	5	5	5	1	5	5	5	0	5	0	5	5	0	5	5	0	0	0	0	0	0	0	0	0

Composition of the strip : ConTRoL, GLYceroL, ERYthritol, D-ARAbinose, L-ARAinose, RIBose, D-XYLose, L-XYLose, ADOnitol, ß-Methyl-D Xyloside, GALactose, GLUcose, FRUctose, MaNnosE, SorBosE, RHAmnose, DULcitol, INOsitol, MANitol, SORbitol, α-Methyl-D Mannoside, α-Methyl-D Glucoside, N-Acetyl Glucosamine, AMYgdalin, ARButin, ESCulin, SALicin, CELiobiose, MALtose, LACtose, MELibiose, SACcharose, TREhalose, INUlin, MeLeZitose, RAFfinose, STArch, GLycoGen, XyLiTol, GENtiobiose, D-TURanose, D-LYXose, D-TAGatose, D-FUCose, L-FUCose, D-ARabitoL, L-ARabitoL, GlucoNaTe, 2-KetoGluconate, 5-KetoGluconate.

Interpretation of results : 0 = negative ; 1 = slightly positive ; 3 = positive ; 5 = strongly positive.

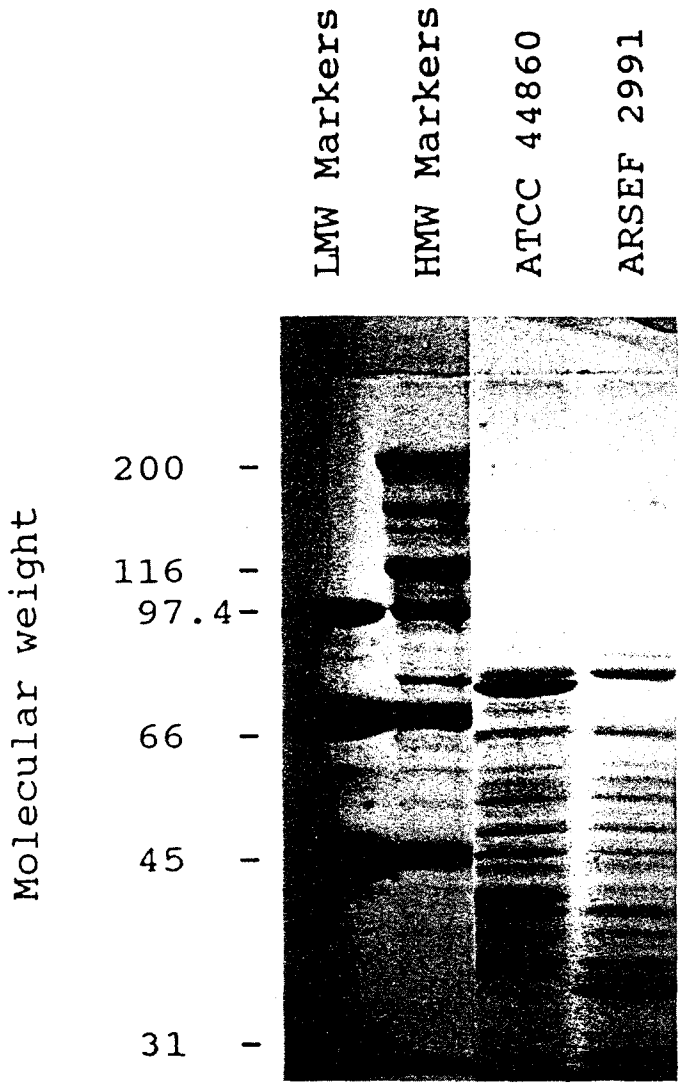

Fig. 1. Protein profiles of two *B. bassiana* strains on sodium dodecyl sulfate-polyacrylamide gel ;
lanes 1 and 2 - molecular weight markers ;
lanes 3 and 4 - strains ATCC 44860 and ARSEF 2991

P = 0.0001) (fig. 2). Strain ATCC 44860 caused 29.5 % larvae mortality at the 10^4 blsp/ml dose ($\chi^2 = 20.76$; P = 0.0001). This strain caused 90.9 % and 98.9 % mortality at concentrations of 10^6 blsp/ml ($\chi^2 = 99.85$; P = 0.0001) and 10^8 blsp/ml ($\chi^2 = 117.23$; P = 0.0001), respectively. Strain ARSEF 2991 showed a weaker toxicity by causing mortality rates of 37.6 % ($\chi^2 = 27.84$; P = 0.0001), 52.9 % ($\chi^2 = 43.08$; P = 0.0001) and 67.1 % ($\chi^2 = 60.68$;

$P = 0.0001$), at concentrations of 10^4, 10^6 and 10^8 blsp/ml, respectively (fig. 2). No significant effect was observed for either strain at a concentration of 10^2 blsp/ml ($P = 0.05$).

The two strains gave rise to similar effects on *L. decemlineata* larvae at concentrations of 10^2 and 10^4 blsp/ml (χ^2 ; df = 3 ; $P = 0.05$). At concentrations of 10^6 and 10^8 blsp/ml strain ATCC 44860 showed a higher toxicity than strain ARSEF 2991 (χ^2 ; df = 3 ; $P = 0.0001$).

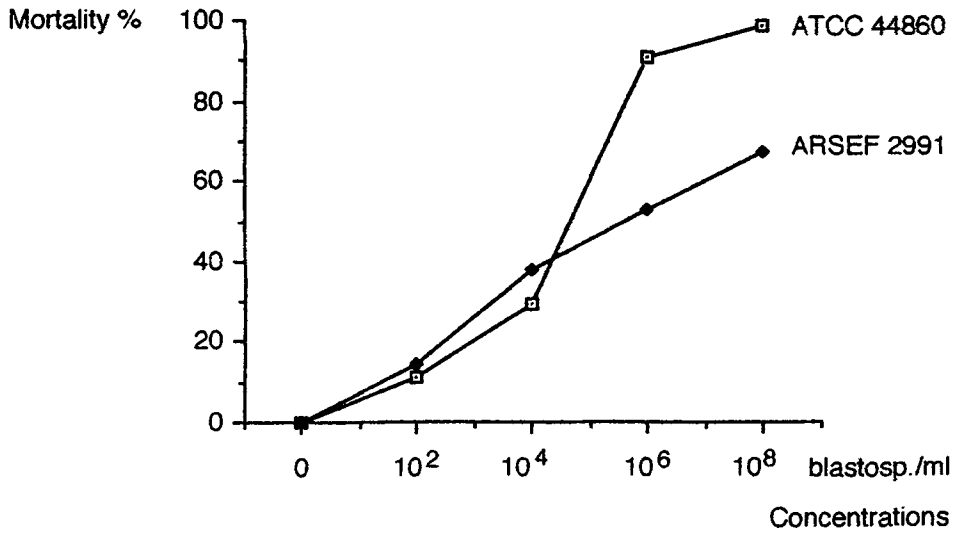

Fig. 2. Mortality, after 10 days, of *L. decemlineata* subjected to different doses of *B. bassiana*

TOXICITY OF THE TWO *B. BASSIANA* STRAINS ON *C. MACULATA* LARVAE

Every tested concentration of strain ARSEF 2991 caused heavy mortality among young larvae which had consumed contaminated pollen (fig. 3). The slope of the mortality-concentration curve is very pronounced at a concentration of 10^2 blsp/ml, and 55.6 % of the larvae died during the first 10 days following treatment ($\chi^2 = 35.71$; df = 3 ; $P = 0.0001$). Concentrations of 10^4 and 10^8 blsp/ml caused 66.7 % mortality while a concentration of 10^6 blsp/ml caused 77.8 % mortality of individuals. The effect did not differ significantly among all doses used ($P = 0.05$) but was significantly different relative to the control ($P = 0.0001$).

Strain ATCC 44860 gave rise to little mortality in *C. maculata* larvae (fig. 3). Concentrations of 10^2, 10^4 and 10^6 blsp/ml did not differ significantly from the control ($\chi^2 = 3.33$; $P = 0.07$), causing 11.1 % mortality. The 10^8 blsp/m concentration was significantly more toxic than the control resulting in 28.7 % mortality ($\chi^2 = 10.00$; $P = 0.002$), but did not differ from the 10^2, 10^4 and 10^6 blsp/ml concentrations ($\chi^2 = 2.00$; $P = 0.16$).

The toxicity of the two *B. bassiana* strains, however, were very different relative to each other in regard to all doses used ($P = 0.001$).

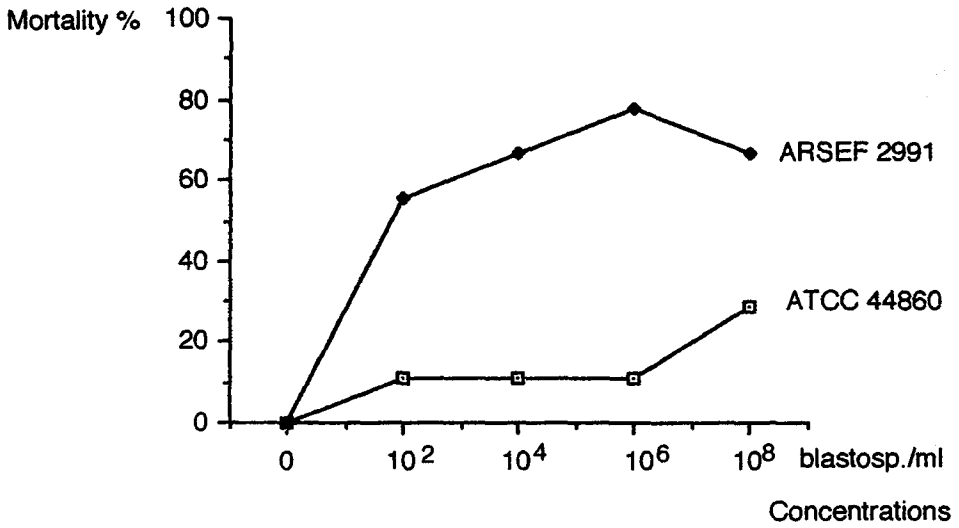

Fig. 3. Mortality, after 10 days, of *C. maculata* subjected to different doses of *B. bassiana*

DISCUSSION

Two *B. bassiana* strains were compared and differentiated, based firstly, on their differential use of carbon sources as determined on API Rapid CH* strips and, secondly on their protein profiles on sodium dodecyl sulfate-polyacrylamide gels.

The ARSEF 2991 strain possesses a capacity to acidify a wider variety of substrates. Following protein analysis, the two strains were distinguished by the presence of different protein bands.

It seems then, that the two strains are very different with respect to biochemical reactions and protein profiles. It was further necessary to determine whether the two strains presented different toxicities to *L. decemlineata* and *C. maculata*.

The toxicity study of the different *B. bassiana* strains on the insects shows a selectivity by the fungus that varies according to the host strain. Some authors report a correlation between the quantity of spores and the cumulative mortality rate (Müller-Kögler, 1967). Other studies (Fargues, 1972) have shown that larvae of the same host can be resistant to certain strains of *B. bassiana* and be very sensitive to other strains of the same pathogen. Often a strain presents no activity on a host while it causes a high mortality rate on other insects of the same family (Fargues, 1976).

Lord *et al.* (1988) have shown that the spotted ladybird beetle is susceptible to certain *B. bassiana* strains. Our study indicates that the *B. bassiana* strains used were both toxic to *L. decemlineata* larvae and yielded a different toxicity on young *C. maculata* larvae. Coccinellid larvae were very susceptible to every concentration of ARSEF 2991 ($P = 0.0001$). However, ATCC 44860 caused negligible mortality in spotted ladybird beetle larvae.

B. bassiana ATCC 44860 presents serious advantages over ARSEF 2991 for use in an integrated pest management program because of its high toxicity towards *L. decemlineata* and its low toxicity towards *C. maculata*. We plan to extend this line of research to a

greater number of *B. bassiana* strains in conjunction with a greater number of insects so as to identify the fungal strains that are potentially toxic to the target insect species and that present minimal negative effects on auxiliaries and predators.

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RÉSUMÉ

Hétérogénéité de deux souches de *Beauveria bassiana* caractérisées par tests biochimiques, profils des protéines et bio-essais sur larves de *Leptinotarsa decemlineata* (Col. : Chrysomelidae) et *Coleomegilla maculata lengi* (Col. : Coccinellidae)

Les profils biochimiques sur galeries API Rapid CH* et les profils protéiques sur gels de polyacrylamide ont été utilisés pour distinguer deux souches du champignon entomopathogène *Beauveria bassiana* (Balsamo) Vuillemin. La toxicité de ces deux souches a été déterminée à des concentrations de 10^2, 10^4, 10^6 et 10^8 blastospores/ml sur des larves du doryphore, *Leptinotarsa decemlineata* Say (Coleoptera : Chrysomelidae) et de la coccinelle maculée *Coleomegilla maculata lengi* Timberlake (Coleoptera : Coccinellidae).

Les deux souches de *B. bassiana* se sont avérées actives à l'égard des larves de *L. decemlineata*. Toutefois la souche ARSEF 2991 s'est avérée pathogène pour les larves de *C. maculata*, alors que la souche ATCC 44860 a provoqué une faible mortalité des larves.

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Current ecological understanding of fungal-like pathogens of fish: what lies beneath?

Rodolphe E. Gozlan[1,2]*, Wyth L. Marshall[3], Osu Lilje[4], Casey N. Jessop[4], Frank H. Gleason[4] and Demetra Andreou[2]

[1] *Unité Mixte de Recherche Biologie des Organismes et Écosystèmes Aquatiques (IRD 207, CNRS 7208, MNHN, UPMC), Muséum National d'Histoire Naturelle, Paris Cedex, France*
[2] *Centre for Conservation Ecology and Environmental Sciences, School of Applied Sciences, Bournemouth University, Poole, Dorset, UK*
[3] *BC Centre for Aquatic Health Sciences, Campbell River, BC, Canada*
[4] *School of Biological Sciences, University of Sydney, Sydney, NSW, Australia*

Edited by:
Télesphore Sime-Ngando, Centre National de la Recherche, France

Reviewed by:
Ryan J. Newton, University of Wisconsin-Milwaukee, USA
Gordon William Beakes, University of Newcastle, UK

****Correspondence:***
Rodolphe E. Gozlan, Unité Mixte de Recherche Biologie des Organismes et Écosystèmes Aquatiques (IRD 207, CNRS 7208, MNHN, UPMC), Muséum National d'Histoire Naturelle, 75231 Paris Cedex, France
e-mail: rudy.gozlan@ird.fr; rgozlan@bournemouth.ac.uk

Despite increasingly sophisticated microbiological techniques, and long after the first discovery of microbes, basic knowledge is still lacking to fully appreciate the ecological importance of microbial parasites in fish. This is likely due to the nature of their habitats as many species of fish suffer from living beneath turbid water away from easy recording. However, fishes represent key ecosystem services for millions of people around the world and the absence of a functional ecological understanding of viruses, prokaryotes, and small eukaryotes in the maintenance of fish populations and of their diversity represents an inherent barrier to aquatic conservation and food security. Among recent emerging infectious diseases responsible for severe population declines in plant and animal taxa, fungal and fungal-like microbes have emerged as significant contributors. Here, we review the current knowledge gaps of fungal and fungal-like parasites and pathogens in fish and put them into an ecological perspective with direct implications for the monitoring of fungal fish pathogens in the wild, their phylogeography as well as their associated ecological impact on fish populations. With increasing fish movement around the world for farming, releases into the wild for sport fishing and human-driven habitat changes, it is expected, along with improved environmental monitoring of fungal and fungal-like infections, that the full extent of the impact of these pathogens on wild fish populations will soon emerge as a major threat to freshwater biodiversity.

Keywords: emerging infectious disease, aquatic, extinction, vertebrate, global, biodiversity, Oomycota, Mesomycetozoea

INTRODUCTION

Fishes are susceptible to diseases caused by a large number of infectious agents including viruses, bacteria, true fungi, fungal-like microrganisms, other protists, and metazoans. This review will briefly discuss true fungal pathogens and then focus on commonly reported zoosporic and amoeboid fungal-like pathogens in the Oomycota and Mesomycetozoea. In general, the number of reported fungal and fungal-like pathogens responsible for diseases in animals is on the increase globally (Fisher et al., 2009; Holdich et al., 2009; Loo, 2009; Frick et al., 2010; Ratnieks and Carreck, 2010; Sarmiento-Ramírez et al., 2010). As such, they are truly emerging diseases with increasing incidence, geographic range, virulence, and some of these fungal and fungal-like pathogens have recently been found in new hosts or are newly discovered (Berger et al., 1998; Brown, 2000; Daszak et al., 2000; Kim and Harvell, 2004; Blehert et al., 2009; Peeler et al., 2010; Cameron et al., 2011). The underpinning drivers of this observed increase remain unclear but these pathogens are known to be opportunistic (Fisher et al., 2012), to have resilient and relatively long-lived environmental stages (Mitchell et al., 2008; Andreou et al., 2009) and may have benefited from recent increase in global trade (Brasier, 2008) and spread of invasive species (Gozlan et al., 2010). Thus increasingly infectious outbreaks are reported in a broad range of species from coral (Kim and Harvell, 2004) to wheat (Wanyera et al., 2006); notable examples include local extinctions of bats (Frick et al., 2010), bees (Ratnieks and Carreck, 2010), turtles (Sarmiento-Ramírez et al., 2010), amphibians (Fisher et al., 2009) and fish (Gozlan et al., 2005, 2009). In aquatic ecosystems fungi and fungal-like pathogen detection in fish hosts is more complicated due to the lack of direct observation of their hosts contrary to frogs or coral, for example (Gozlan, 2012). This is particularly true in freshwater systems where, despite being responsible for pan-continental population extinctions, some diseases caused by fungal and fungal-like pathogens are chronic with no clear external symptoms (Gozlan et al., 2005; Kocan and Hershberger, 2006; Andreou et al., 2011, 2012). This is very well illustrated, for example, by the rosette agent *Sphaerothecum destruens*, which has been rapidly spreading all over Europe via an invasive healthy fish host carrier (Gozlan et al., 2005; Gozlan, 2012). This fungal—like pathogen is intracellular, causing high mortality (up to 90%) after about 20–30 days but it can only be confidently detected by PCR analysis (Mendonca and Arkush, 2004). The paradox is that despite huge pan-continental population extinction, it remains difficult to

characterize the true ecological impact of fungal and fungal-like pathogens on freshwater fish populations.

Despite fisheries representing a key ecosystem service for millions of people around the world, the full appreciation of disease risk associated with fungal pathogen emergence remains limited (Gozlan et al., 2006). Here, we review the current knowledge gaps of fungal microbes in fish, their phylogeography along with the current methods of detection and associated limitations and a global ecological understanding of their impacts on fish host populations. With an increasing volume of fish translocation around the world for farming and sport fishing, the relative absence of fish-infecting fungi outbreaks when compared to other more easily observed taxa exemplifies the current concern of a reporting bias in wild fish populations (Gozlan, 2012).

DIVERSITY AND PHYLOGENY

In recent years interest in the phylogeny of eukaryotes has been re-evaluated (see Adl et al., 2005). Based on data from sequencing genes, particularly rRNA gene sequences, Baldauf (2003) reassigned eukaryotes into eight different branches or supergroups within the tree of life, namely the opisthokonts, amoebozoa, plants, cercozoa, alveolates, heterokonts, discicristates, and excavates. The true fungi and Mesomycetozoea are placed along with the animals in the Opistokonta. The Mesomycetozoea form a clade, which falls on the animal branch, near the animal fungal divide (Paps et al., 2013). All of the Oomycota are placed into the Heterokont supergroup.

True fungi constitute the most species rich group of organisms on earth with 35 recognized classes and 129 orders (Hibbett et al., 2007). The majority of the fungi causing infection in fish belong to the phylum Ascomycota, with thick-walled non-motile spores (Hibbett et al., 2007). Within the Ascomycota, species from several genera have been reported to be associated with fish infections (**Table 1**). In addition to the Ascomycota, species belonging to the (earlier diverging) Zygomycota have also been reported to cause disease. The majority of the fungi, which can cause infection in fish are opportunistic and not exclusive parasites of fish. They are most commonly known as plant pathogens (e.g., *Penicillium corylophilum* and *Phoma herbarum*), soil fungi (e.g., *Paecilomyces lilacinus*) and some have even been reported to cause infection in immunosuppressed humans (e.g., *Exaphiala xenobiota* and *Ochroconis humicola*).

Branching close to the divergence between fungi and animals there is a relatively recently recognized clade of organisms, the Mesomycetozoea (Mendoza et al., 2002; Ragan et al., 1996), which includes a number of species that are pathogenic to aquatic organisms including fish (Mendoza et al., 2002; Glockling et al., 2013). Within the Mesomycetozoea, species can be divided further into the orders of Dermocystida and Ichthyophonida. The Dermocystida include a number of species that can be pathogenic to fish, the most notable being *Sphaerothecum destruens,* which can infect a wide range of hosts and has been shown to cause disease and high mortality in cyprinids (Andreou et al., 2011, 2012) and salmonid species (Arkush et al., 1998; Paley et al., 2012). The order also includes numerous *Dermocystidium* sp., which can infect a variety of fish species (see **Table 1**). The diversity of the *Dermocystidium* genus is probably underestimated as a large proportion of recorded cases in the literature only identify the pathogen to genus level. This can be addressed by applying molecular techniques to identify species. Within the Ichthyophonida, *Ichthyophonus hoferi* is the most common parasite of salt and freshwater fish (Hershberger et al., 2010; Kocan et al., 2010; Gregg et al., 2012; Hamazaki et al., 2013).

The Oomycete parasites of fishes are placed in the Phylum Oomycota and fall into either the saprolegnialean lineage or the peronosporalean lineage. The Oomycetes are water moulds which morphologically resemble fungi, but are taxonomically distinct, encompassing species that are parasitic to a large diversity of host species (Beakes et al., 2012). The majority of the species, which can infect and cause disease in fish belong to the order of Saprolegniales and fall within the genera of *Saprolegnia*, *Aphanomyces* and *Achlya*. A smaller number of species fall within the genus *Pythium*, a member of the peronsporalean lineage. Twelve species of *Saprolegnia* and six species each of *Aphanomyces* and *Achlya* (**Table 1**) are more often described in the literature as causing infection in fish; with the most common pathogens of fish being *Saprolegnia parasitica* and *Aphanomyces invadans* which have relatively high generalist indices (See **Table 1**). *S. parasitica* has been reported to cause disease in 12 fish species whilst *A. invadans* can parasitize 48 fish species.

HOST SPECIFICITY

A common characteristic of the fish pathogens within Fungi, Mesomycetozoea, and Oomycetes is their generalist nature, with the majority of species infecting and causing disease in fishes across different families (**Table 1**). All three groups include an equal proportion of species with generalist indices above 3 indicating that they are true generalists (Poulin and Mouillot, 2003). Due to higher reporting and detection of disease in farmed environments, most disease reports are from aquaculture facilities and involve cultured fish species. There is thus a bias in the fish species reported as susceptible to these pathogens and a possible underestimation of their generalist nature (Ramaiah, 2006). A large number of species have a single record of affecting a single fish species in the literature and thus the generalist index cannot be calculated.

The ability of fungal and fungal-like pathogens to infect multiple hosts ("the widest spectrum of host ranges for any group of pathogens" according to Fisher et al., 2012; see **Table 1** for fish). often drives high virulence in the most susceptible hosts (Andreou et al., 2012; Huchzermeyer and Van der Waal, 2012). The aspect of generalism in pathogenicity is important due to the fact that generalist pathogens are more likely to emerge through host switching (Woolhouse and Gowtage-Sequeria, 2005), and it is often overlooked (Yamamoto and Kilistoff, 1979; Peeler et al., 2010). However, it is commonly accepted (Ewald, 1994) that in single hosts the optimum level of virulence is determined by the trade-off between virulence and transmissibility (Davies et al., 2001). Thus, the composition of the community and the susceptibility of each host could alter its potential transmissibility and the outcome of infection (Woolhouse et al., 2001). Experimental challenges to fungal and fungal-like pathogens of several fish host species are currently needed. This could involve simple one host – one pathogen challenges such as in Andreou et al. (2012) or a

Table 1 | List of Fungi, Mesomycetozoea and Oomycetes species, which have been recorded as fish parasites in the Web of Knowledge since 1997.

Species	Order	Reported hosts	Generalist index	References
FUNGI				
Cladosporium sphaerospermum	Capnodiales	*Lutjanus campechanu*	NA	Blaylock et al., 2001
Exophiala angulospora	Chaetothyriales	*Gadus morhua*	NA	Gjessing et al., 2011
Exophiala pisciphila	Chaetothyriales	*Stegostoma fasciatum*	NA	Marancik et al., 2011
Exophiala xenobiotica	Chaetothyriales	*Pseudocaranx dentex*	NA	Munchan et al., 2009
Paecilomyces lilacinus	Eurotiales	*Clarias gariepinus* *Oreochromis niloticus niloticus* *Tilapia aurea*	2	Rand et al., 2000a,b; Ali et al., 2011
Penicillium corylophilum	Eurotiales	*Lutjanus campechanus*	NA	Blaylock et al., 2001
Ochroconis humicola	Incertae sedis	*Pseudocaranx dentex* *Pagrus major* *Sebastiscus marmoratus*	3.3	Wada et al., 2005; Munchan et al., 2009
Mucor circinelloides	Mucorales	*Pelteobagrus fulvidraco* *Pseudocaranx dentex*	5	Ke et al., 2010; Marancik et al., 2011
Phoma herbarum	Pleosporales	*Clarias gariepinus* *Oncorhynchus tshawytscha* *Oreochromis niloticus niloticus*	4	Faisal et al., 2007; Ali et al., 2011
Phialemonium dimorphosporum	Sordariales	*Mugil cephalus*	NA	Sosa et al., 2007a,b
Ochroconis humicola	Incertae sedis	*Pseudocaranx dentex* *Pagrus major* *Sebastiscus marmoratus*	3.3	Wada et al., 2005; Munchan et al., 2009
MESOMYCETOZOEA				
Dermocystidium cyprini	Dermocystida	*fluviatilis* *Gymnocephalus cernuus* *Cyprinus carpio*	3.3	Lotman et al., 2000; Pekkarinen and Lotman, 2003
Dermocystidium fennicum	Dermocystida	*PercaPerca fluviatilis*	NA	Pekkarinen and Lotman, 2003
Dermocystidium koi	Dermocystida	*Cyprinus carpio*	NA	Gjurcevic et al., 2008
Dermocystidium percae	Dermocystida	*Perca fluviatilis*	NA	Morley et al., 2008
Dermocystidium branchiale	Dermocystida	*Salvelinus alpinus* *Salmo trutta*	2	Kristmundsson and Richter, 2009
Sphaerothecum destruens	Dermocystida	*Abramis brama* *Cyprinus carpio* *Leucaspius delineatus* *Oncorhynchus kisutch* *Oncorhynchus mykiss* *Oncorhynchus tshawytscha* *Pseudorasbora parva* *Rutilus rutilus* *Salmo salar* *Salmo trutta* *Salvelinus fontinalis*	3.6	Arkush et al., 1998; Gozlan et al., 2005; Andreou et al., 2012; Paley et al., 2012
Ichthyophonus hoferi	Ichthyophonida	*Citharichthys stigmaeus* *Clupea harengus* *Clupea pallasi* *Hypomesus pretiosus* *Microgadus proximus* *Oncorhynchus kisutch* *Oncorhynchus mykiss* *Oncorhynchus tshawytscha* *Pleuronectes flesus* *Salmo trutta* *Sebastes alutus* *Sebastes emphaeus* *Sebastes flavidus* *Sprattus sprattus*	3.6	Rahimian, 1998; Criscione et al., 2002; Hershberger et al., 2002; Schmidt-Posthaus and Wahli, 2002; Gavryuseva, 2007; Kocan et al., 2010; Kramer-Schadt et al., 2010; Rasmussen et al., 2010; Gregg et al., 2012; Hamazaki et al., 2013

(Continued)

Table 1 | Continued

Species	Order	Reported hosts	Generalist index	References
Ichthyophonus irregularis	Ichthyophonida	*Limanda ferruginea*	NA	Rand et al., 2000a,b
OOMYCETES				
Achlya bisexualis	Saprolegniales	*Mugil cephalus*	NA	Sosa et al., 2007a
Achlya klebsiana	Saprolegniales	*Oreochromis niloticus niloticus*	2.3	Ali et al., 2011; Cao et al., 2013
		Clarias gariepinus		
		Pelteobagrus fuvidraco		
Achlya americana	Saprolegniales	*Coregonus lavaretus holsatus*	NA	Czeczuga et al., 2004
Achlya. oblongata	Saprolegniales	*Coregonus lavaretus holsatus*	NA	Czeczuga et al., 2004
Achlya racemosa	Saprolegniales	*Odonthestes bonariensis*	NA	Pacheco Marino et al., 2009
Achlya ambisexualis	Saprolegniales	*Oncorhynchus mykiss*	NA	Vega-Ramirez et al., 2013
Aphanomyces parasiticus	Saprolegniales	*Coregonus lavaretus holsatus*	NA	Czeczuga et al., 2004
Aphanomyces frigidophilus	Saprolegniales	*Coregonus lavaretus holsatus*	2	Czeczuga et al., 2004, 2005
		Salmo trutta		
Aphanomyces invadans	Saprolegniales	*Alosa sapidissima*		
		Anguilla anguilla		
		Ameiurus melas		
		Archosargus probatocephalus		
		Bairdiella chrysoura		
		Brevoortia tyrannus		
		Brycinus lateralis		
		Barbus poechii		
		Barbus paludinosus		
		Barbus unitaeniatus		
		Catla catla		
		Channa marulius		
		Clarias gariepinus		
		Clarias ngamensis		
		Cyprinus carpio		
		Fundulus heteroclitus		
		Fundulus majalis		
		Hepsetus odoe		
		Hydrocynus vittatus		
		Ictalurus punctatus		
		Leiopotherapon unicolor		
		Labeo lunatus		
		Labeo cylindricus		
		Lepomis macrochirus		
		Macquaria ambigua		
		Maccullochella peelii		
		Marcusenius macrolepidotus		
		Micralestes acutidens		
		Micropterus salmoides		
		Mugil cephalus		
		Mugil curema		
		Nematalosa erebi		
		Oncorhynchus mykiss		
		Oreochromis andersonii		
		Oreochromis macrochir		
		Petrocephalus catostoma		
		Pharyngochromis acuticeps		
		Pogonias cromis		
		Sargochromis codringtonii		
		Sargochromis giardi		
		Serranochromis robustus		
		Serranochromis angusticeps		
		Serranochromis macrocephalus		
		Schilbe intermedius		
		Silurus glanis		
		Tilapia sparrmanii		

(Continued)

Table 1 | Continued

Species	Order	Reported hosts	Generalist index	References
Tilapia rendalli		*Trinectus maculates*	3.7	Thompson et al., 1999; Hawke et al., 2003; Harikrishnan et al., 2005; Kiryu et al., 2005; Webb et al., 2005; Vandersea et al., 2006; Sosa et al., 2007b; Oidtmann et al., 2008; Saylor et al., 2010; Boys et al., 2012; Go et al., 2012; Huchzermeyer and Van der Waal, 2012; Saikia and Kamilya, 2012
Aphanomyces irregularis	Saprolegniales	*Coregonus lavaretus holsatus*	NA	Czeczuga et al., 2004
Aphanomyces laevis	Saprolegniales	*Aplocheilus panchax* *Clarias gariepinus* *Oreochromis niloticus niloticus*	4	Mondal and De, 2002; Ali et al., 2011
Aphanomyces salsuginosus	Saprolegniales	*Salangichthys microdon*	NA	Takuma et al., 2010
Saprolegnia australis	Saprolegniales	*Oncorhynchus nerka* *Plecoglossus altivelis* *Salmo trutta*	3.3	Hussein et al., 2001; Chang et al., 2002; Fregeneda-Grandes et al., 2007
Saprolegnia brachydanis	Saprolegniales	*Danio rerio*	NA	Ke et al., 2009a,b
Saprolegnia diclina	Saprolegniales	*Acipencer persicus* *Oncorhynchus mykiss Salmo salar* eggs *Salmo trutta* *Sciaenops ocellatus*	3.3	Leano et al., 1999; Fregeneda-Grandes et al., 2007; Ghiasi et al., 2010; Shahbazian et al., 2010; Thoen et al., 2011
Saprolegnia ferax	Saprolegniales	*Carassiuus auratus* *Coregonus lavaretus holsatus* *Odonthestes bonariensis* *Oncorhynchus mykiss* eggs *Salmo trutta*	3.6	Czeczuga et al., 2004; Fregeneda-Grandes et al., 2007; Ke et al., 2009a,b; Pacheco Marino et al., 2009; Shahbazian et al., 2010; Cao et al., 2013
Saprolegnia furcata	Saprolegniales	*Salmo trutta*	NA	Fregeneda-Grandes et al., 2007
Saprolegnia hypogyana		*Oncorhynchus mykiss* eggs *Salmo trutta*	2	Fregeneda-Grandes et al., 2007; Shahbazian et al., 2010
Saprolegnia parasitica	Saprolegniales	*Acipencer persicus* *Astyanax eigenmanniorum* *Astyanax fasciatus* *Bidyanus bidyanus* *Coregonus lavaretus holsatus* *Ictalurus punctatus* *Odontesthes bonariensis* *Oncorhynchus mykiss* *Oncorhynchus masu*		
		eggs *Oncorhynchus nerka* *Salmo salar* eggs *Salmo trutta* *Salvelinus leucomaenis*	3.3	Bangyeekhun et al., 2001; Hussein and Hatai, 2002; Czeczuga et al., 2004; Fregeneda-Grandes et al., 2007; Mancini et al., 2008, 2010; Mifsud and Rowland, 2008; Ghiasi et al., 2010; Shahbazian et al., 2010; Thoen et al., 2011

(Continued)

Table 1 | Continued

Species	Order	Reported hosts	Generalist index	References
Saprolegnia polymorpha	Saprolegniales	*Cyprinus carpio*	NA	Willoughby, 1998
Saprolegnia salmonis	Saprolegniales	*Coregonus lavaretus holsatus* *Oncorhynchus masu* *Oncorhynchus mykiss* *Oncorhynchus nerka* *Plecoglossus altivelis* *Salmo trutta* *Salvelinus leucomaenis*	2.4	Hussein et al., 2001; Chang et al., 2002; Hussein and Hatai, 2002; Czeczuga et al., 2004, 2005
Saprolegnia shikotsuensis	Saprolegniales	*Coregonus lavaretus holsatus*	NA	Czeczuga et al., 2005
Pythium aquatile	Pythiales	*Coregonus lavaretus holsatus*	NA	Czeczuga et al., 2004
Pythium pulchrum	Pythiales	*Coregonus lavaretus holsatus*	NA	Czeczuga et al., 2004
Pythium thalassium	Pythiales	*Coregonus lavaretus holsatus*	NA	Czeczuga et al., 2004
Pythium torulosum	Pythiales	*Coregonus lavaretus holsatus*	NA	Czeczuga et al., 2004

A generalist index was calculated for each parasite using the method described in Poulin and Mouillot (2003); where species with two or more hosts can have generalist indices ranging from 1 (all host species share the same genus) to 5, using the five taxonomic levels of genus, family, order, class, and phylum. NA stands for non-applicable as the index cannot be calculated when only one host has been reported. The fish taxonomy proposed by Nelson (1994) was used in calculating all generalist indices.

combination of multi-hosts challenges. In addition, experimental data on the free-living elements of these life cycles of pathogens such as the presence of zoospores, would allow the measurement of their production, longevity in the system and their resistance to a range of abiotic factors (e.g., temperature, PH). These data are needed to build reliable models to test host susceptibility, understand the controlling factors of infectious phase as well as the recovery phase typical of SIT or SEIR epidemiological models (susceptible-exposed-infectious-recovered).

LIFE CYCLES AND STAGES

In the assimilative phases of oomycetes and most of the true fungi, colonization of new tissues is accomplished through the growth of hyphae, with the exception of the black yeasts, *Exophiala*, which may transition between yeast and hyphal forms (dimorphism) (de Hoog et al., 2011). Mesomycetozoeans more often grow as round multinucleate coenocytes. These can be concentrated in visible cysts in the genus *Dermocystidium* (e.g., Lotman et al., 2000) or disseminated or nodular in *S. destruens* and *Ichthyophonus* (Sindermann and Scattergood, 1954; Arkush et al., 1998). Hyphal forms have been described in some *Dermocystidium* (Dykova and Lom, 1992) and are common in *Ichthyophonus* (Sindermann and Scattergood, 1954; Rand, 1994; Franco-Sierra and Alvarez-Pellitero, 1999). Only true fungi, however, have septate hyphae, although some oomycetes have segmented or plugged thalli and thus are also compartmentalized.

All fungal and fungal-like pathogens have prolific asexual reproduction (r-strategy) functioning for dispersal or further dissemination within the host. In the fungi, this is through the production of conidiospores (Ascomycota) or sporangiospores (Zygomycota), and the budding of yeast stages. These spores are not motile and are protected by a chitinous cell wall. The durability and resilience of these spores is an important adaptation for increasing opportunities to encounter new susceptible hosts (Fisher et al., 2012). These spores can survive in a dormant state during conditions unfavorable for growth. Oomycetes produce biflagellated zoospores within sporangia, usually located either at the terminal ends of hyphae. These spores function to disperse the parasite between hosts and typically encyst after a short period of motility. In *Saprolegnia* species longer lived secondary zoospores emerge from cysts produced by primary zoospores. This pattern of re-emergence called polyplanetism, may be repeated several times (Bruno et al., 2011), and most likely functions to allow several opportunities to contact a new host. Zoospores of many oomycetes are chemotactic, responding to amino acids, carbohydrates and a range of aldehyde attractants (Donaldson and Deacon, 1993). The encysted zoospores of *S. parasitica* are decorated by long hooked hairs that are thought to aid in attachment to the fish host (Van West, 2006; Walker and Van West, 2007).

Reproduction in the Mesomycetozoea is more varied (Mendoza et al., 2002). *S. destruens* produce non-motile walled endospores which may either infect other cells within the same host or spread and infect a new host (Arkush et al., 2003). Endospores also produce singly flagellate zoospores upon exposure to fresh water (Arkush et al., 2003) but it is not clear whether these zoospores are infective (Paley et al., 2012). *Dermocystidium* has similar development with zoospore development within spores, but zoospores are infective (Olson et al., 1991). Released endospores of both *Dermocystidium salmonis* and *S. destruens* have the capacity to release zoospores for several weeks at 4°C (Olson et al., 1991; Andreou et al., 2009). The life cycle of *Ichthyophonus* is less understood and varies with pH (Okamoto et al., 1985; Spanggaard et al., 1995; Franco-Sierra and Alvarez-Pellitero, 1999). Single and multinucleate endospores are produced in culture and *in vivo* (Okamoto et al., 1985; Spanggaard et al., 1995; Franco-Sierra and Alvarez-Pellitero, 1999). Motile zoospores are not produced but amoeboid stages are released under specific pH optima in culture (e.g., Okamoto et al., 1985). Transmission is also not well understood, except

that the parasite can be acquired through carnivory (Jones and Dawe, 2002). Kocan et al. (2013) describe small amoeboid stages within the stomach wall of sculpin and trout hosts after feeding of infected tissues and hypothesize that these amoebae represent the infectious stage. The infectious stage of planktivorous fish is still unknown (Gregg et al., 2012) and an alternate host is suspected (Sindermann and Scattergood, 1954).

In parasites of fishes sexual reproduction (s-strategy) has only been described in a few oomycetes. When sexual reproduction occurs, the two dissimilar gametangial structures called the oogonium and the antheridium grow closer together until they fuse, and haploid nuclei from the antheridia fertilize the eggs within the oogonia forming diploid oospores. In free living oomycetes the fertilized zygote, or oospore, is typically resistant and can survive for prolonged periods. Meiosis and recombination occur before germination of the oospore. However, the main oomycete pathogens of live fish (e.g., *A. invadans* and *S. parasitica*), do not generally (in case of *A. invadans* never) reproduce sexually and therefore rely entirely on asexual zoosporogenesis (r-strategy). Some egg infecting species do produce oogonia (e.g., *S. australis*, *S. diclina*, *S. ferax*) but even in these species oospore germination is rarely if ever observed. It is highly unlikely that oospores serve as effective resistant survival structures for fish parasitic oomycetes. Most true fungal parasites of fish are described as "fungi imperfecti," based on the lack of a described sexual stage.

TROPHIC MODES

Research on animal parasites has revealed that many of these species are not exclusively saprophytic or parasitic (Gleason et al., 2010; McCreadie et al., 2011). In fact, their precise ecological functions can only be understood with intensive metagenomic investigations, which have rarely been conducted (Jiang et al., 2013). Nonetheless, these microorganisms are frequently characterized as either saprotrophs or biotrophs (Gleason et al., 2010; McCreadie et al., 2011). Saprotrophs usually do not infect live hosts, rather they grow on non-living organic material. In contrast many biotrophs cannot grow outside the host, but some can be grown in culture. Growth of parasites in culture allows research on mechanisms of infection and sequencing genes. Facultative parasites can grow well as either parasites or saprotrophs. Many eukaryotic microorganisms are thought to be parasites primarily because they cannot be grown outside their host, but in fact their trophic relationships remain to be determined. Many Oomycete species are primarily saprotrophs, yet few can become parasites under certain conditions, such as compromised immunity in their hosts. The important point is that they have alternative substrates for growth outside the host, which is an important characteristic of emerging infectious diseases (EID) (Fisher et al., 2012).

PROTEINS AS SUBSTRATES FOR GROWTH

For a long time proteins have been known to be good substrates for the isolation of Oomycetes into pure culture and for their subsequent growth in liquid media (Sparrow, 1960). For example, casein and keratin can be useful substrates for isolation and growth. Furthermore animal hosts and tissues are known to be protein rich environments. Czeczuga et al. (2002) isolated many species of Oomycetes from specimens of fish muscles placed in freshwater lakes. Some of these specimens came from fish, which were known to be hosts for Oomycetes. Smith et al. (1994) demonstrated proteolytic activity of *Saprolegnia diclina, ferax,* and *parasitica* by observing the clearing of casein on solid media. Proteins must be digested extra-cellularly and the amino acids produced must be transported into the cell prior to their catabolism. Jiang et al. (2013) documented the presence of genes for serine, metallo- and cysteine proteases and genes for amino acid transporters in the complete sequence of the genome of *S. parasitica*.

Saprotrophic isolates of *Saprolegnia*, *Achlya*, *Dictyuchus*, *Leptolegnia*, *Aphanomyces*, *Apodachlya,* and *Pythium* grew rapidly on many but not all amino acids as sole sources of carbon and nitrogen in liquid media (Gleason et al., 1970a,b; Faro, 1971). Alanine, proline, glutamate, aspartate, leucine, lysine, arginine, serine, and phenylalanine were especially good carbon sources, there was very little or no growth on valine, isoleucine, threonine, methionine, and glycine, and there were considerable differences in rates of utilization among the species tested. Saprotrohic and parasitic isolates of *Saprolegnia* can remove all amino acids from liquid media during growth on mixtures of amino acids (Gleason, 1973; Nolan, 1976). These data indicate that many Oomycetes have the capacity for digestion of proteins and subsequent uptake and catabolism of amino acids. Therefore they commonly grow in protein rich environments. Recently, a few species in the Mesomycetozoea have been grown in culture (Glockling et al., 2013), but nutritional experiments have not been conducted, and little is known about their proteolytic capacities.

CURRENT DETECTION TECHNIQUES

Lesions formed by parasites were initially characterized from phenotypic, serological and morphological properties of the pathogen. Isolation and culturing of causative organisms from swabbed lesions of infected fish has been an integral part in understanding the taxonomic groupings, etiology of the disease, infectivity, and host-parasite relationships. The process of isolating and identifying pathogens can however be a time consuming process requiring a high level of technical expertise.

Morphological identification of microbial species, which often requires identification of reproductive stages, is difficult to accomplish directly from ulcerated tissue. Direct visualization of pathogens in infected tissues has been made possible with the development of species-specific fluorescent probes. For example, the monoclonal antibody MAb 3gJC9, which is specific for an antigen involved in the pathogenicity of *Aphanomyces astaci* and *A. invadans (=piscicida)* in infected crayfish and fish respectively, has been used for immunofluorescent identification of these species in infected tissues (Miles et al., 2003). The approach was found to be more sensitive than the conventional staining method, Grocott's methanamine silver stain, in that it enabled the detection of early stages of infection (Grocott, 1955; Miles et al., 2003). Fluorescent hybridization (FISH) probes have also been used to identify specific pathogens in infected tissues *in situ*. For example, *A. invadans* was found to be a primary oomycete pathogen in ulcerative mycosis of infected estuarine fish in North Carolina and Florida using a FISH assay (Vandersea et al., 2006; Sosa et al., 2007a,b). Continuing improvements in

isolation, culturing and *in situ* approaches is essential for broadening our understanding of disease pathology and etiology and more fundamentally the morphology and physiology of these pathogenic species. In comparison to morphological and physiological classification, the rapid advances in molecular techniques has improved the reliability and accuracy of the tool in distinguishing many taxa, such as the microsporidian taxa (Larsson, 2005). A molecular approach has also led to rapid development of diagnostic tools which involve polymerase chain reaction (PCR), amplification of nucleic acids, restriction enzyme digestion, probe hybridization and nucleotide sequencing. The development of the FISH assay for example was as a result of using a sensitive PCR technique. The use of PCR to detect and identify infections has become commonplace (Tsui et al., 2011). A large number of disease-causing pathogens are often identified to genus level (e.g., *Dermocystidium* sp.) and not species level. The number of species being identified has been constantly increasing through the use of molecular tools for disease detection and identification. A concerted effort to use the same DNA loci would increase the available genetic information resulting in a better resolution of the phylogenetic relationships within and between these groups. The 18S rRNA gene has been used extensively (for Fungi and Mesomycetozoans); however the Internal Transcribed Sequence 1 (ITS1) has been more extensively used within the Oomycetes. As documented by Diéguez-Uribeondo et al. (2007) for the *S. diclina- S. parasitica* complex, both molecular and morphological and physiological data can help solve phylogenetic relationships. Thus, using ITS rRNA gene, five phylogenetic separate clades were identified for the *Saprolegnia* complex, with all isolates collected from salmonid lesions falling into a single clade (i.e., clade I). However, within that clade I, parasitic isolates came from a wide range of hosts including, for example, crustaceans, and catfish but also non-pathogenic isolates from soil and water. Molecular analyses have the potential to discriminate at the subspecies or strain level (Phadee et al., 2004). The level of sensitivity of the molecular techniques in the clinical context has however been sporadic (Cunningham, 2002). This is largely due to the relatively low genomic information that is available through public data bases such as Genbank. We propose that all reported cases of disease outbreaks should have both of these regions sequenced and reported within the literature. The use of these loci will allow both detection at species level (18S rRNA) and identification of different strains (using ITS1) within the same species; allowing for a better identification and detection of virulent strains. This collection of information alongside morphological and physiological data will increase the resolution of the phylogenetic information and the sensitivity of molecular identification.

Alternative detection approaches include loop-mediated isothermal amplification (LAMP) and pyrolysis mass spectrometry. LAMP has the potential of increasing sensitivity of pathogen compared to PCR and unlike PCR it is not inactivated by tissue and blood-derived inhibitors or genomic DNA (Savan et al., 2005). It has been used in the detection of trypanosome infection (Savan et al., 2005). Pyrolysis mass spectrometry profile and canonical variate analysis have been used to demonstrate clusters of *A. invadans* isolates and discriminate them from non-pathogenic *Aphanomyces* species (Lilley et al., 2001). The development and refinement of multiple approaches of detection have their place in increasing the knowledge of the pathogen, its distribution, impact and possible management.

IMPORTANCE OF FUNGAL PATHOGENS IN AQUACULTURE

Fish represent a key ecosystem service for fisheries and aquaculture across the world (Zhao et al., 2014). The annual harvest is about 42 million tonnes (marine and freshwater) and the sector employs 33.1 million people, highlighting the tremendous social cost of fisheries (Gozlan and Britton, 2014). The livelihoods of 60 million people in the developing world are dependent on river fisheries and millions more rely on them for food (Dugan and Allison, 2010). However, disease in aquaculture represents the most significant economic losses and in particular fungal infections, which in terms of economic impact are second only to bacterial diseases (Neish and Hughes, 1980; Noga, 1993; Bruno et al., 2011; Ramaiah, 2006; Van West, 2006; Gonçalves and Gagnon, 2011). For example it has been reported in Japan some annual losses of 50% in the production of coho salmon *Oncorhynchus kisutch* and elvers of eel *Anguilla Anguilla* due to outbreaks of *S. parasitica* (Hatai and Hoshai, 1994; Scarfe, 2003).

In the last decades, the aquaculture sector has seen a change in the fish production with a trend toward intensification with the use of recirculating systems (Larkin and Sylvia, 1999). The underpinning drive was a reduction of environmental footprint, a better control of the rearing environment and increased biosecurity. Nonetheless, this improved control of rearing conditions, has lead the industry to also increase the stocking densities of target fish. Thus, it has resulted in an increase of disease outbreaks, with faster transmission and increased mortalities (Bondad-Reantaso et al., 2005; Whittington and Chong, 2007; Peeler et al., 2010; Gonçalves and Gagnon, 2011).

One of the key risks associated with this new aquaculture environment is the stress caused by intensive production. Some fungal pathogens such as *Saproglegnia* for example are more prevalent and virulent in host (salmonids in particular) that are raised under stressful conditions (Willoughby and Pickering, 1977; Willoughby, 1978; Jeney and Jeney, 1995). However, other significant pathogen risks in aquaculture arise from the large and frequent movement of young stages due to either a lack of or insufficient national production, or due to fish species for which the life cycle has not yet been mastered at a commercial level, or even contaminated sources of water supplies. Of course this is not specific to fungal or fungal-like pathogens but their generalist and opportunistic nature associated with a wide environmental tolerance are risk factors that may lead to significant loss of production (Harrell et al., 1986; Paley et al., 2012).

In addition, mycoses spread in fishes are often seen as a secondary phenomenon. However, due to their virulence, their current emergence in wild fish populations and also the risk of spill back from aquaculture facilities to the wild, routine pathological examination should include (in addition to bacteriological ones) mycological examination (see Rehulka, 1991 for details). Dominant fungal pathogens reported in aquaculture are oomycetes including the genera *Achlya*, *Aphanomyces* and *Saprolegnia* (Willoughby and Pickering, 1977; Blazer and

Wolke, 1979; Noga, 1993). *A. invadans* for example can cause epizootic ulcerative syndrome in over a hundred of mostly freshwater fish (e.g., Vishwanath et al., 1998; Blazer et al., 2013; Nsonga et al., 2013) but also in some brackish fish species (Catap and Munday, 2002; Sosa et al., 2007b). In aquaculture conditions, the most appropriate control is through eradication of the stock, quarantine of new stocks and good husbandry (Scarfe, 2003; Whittington and Chong, 2007) and as such represent a significant cost to the trade (Forneris et al., 2003).

There are no treatments that are specific to fungal and fungal-like pathogens but existing ones such as the use of hydrogen peroxide or formalin (Arndt et al., 2001), malachite green (Van West, 2006), sodium chloride (Schreier et al., 1996) and bronopol (Shinn et al., 2012) all present some significant issues related to either human or fish health or to efficacy of the treatments (Carana et al., 2012). Malachite green was banned by the US and EU in early nineties and since then formalin has probably been the most effective control measure but there is a strong possibility that this will soon also be banned from use. Other treatment such as bronopol and other agents are not as effective. Other treatments such as the use of ozone in recirculating systems have to be specifically adapted for fugal pathogens. For example, studies have shown that ozone treatments for *Saprolegnia* are effective with doze from about 0.01 to 0.2 mg.L^{-1} (Gonçalves and Gagnon, 2011) but present a cost of through reduction in hatching rates (e.g., 42.6–49.1%). New treatments based on plant extract have shown some promising paths but further evaluations need to be performed before its use by the industry (Carana et al., 2012).

ECOLOGICAL IMPACT ON WILD POPULATIONS

The emergence of infectious diseases caused by fungal and fungal-like microbes continues to negatively impact wild fish populations, leading in some cases to local and pan-continental extinctions (Gozlan et al., 2005, 2010; Rowley et al., 2013). Thus, understanding of the true ecological cost of fungal and fungal-like microbes is pivotal to improve our conservation practices of fish populations, especially freshwater species, as declines in populations, species distributions and species diversity continue to occur at alarming rates (Myers, 1993; Singh, 2002; Romansic et al., 2009).

Fungal and fungal-like microbes that cause disease emergence in wild fish (**Table 2**), crayfish, amphibians and other aqutic taxa include *Saprolegnia, Batrachochytrium, Ichthyophonous, Aphanomyces, Achyla* and *Sphaerothecum* (Bruno et al., 2011; Swei et al., 2011). *Saprolegnia* and *Sphaerothecum* spp. are impacting wild salmon populations around the world (Willoughby et al., 1983; Van West, 2006; Andreou et al., 2009), prevailing in 32% of adult late-fall-run chinook salmon returning to Battle Creek on the Upper Sacramento River (Arkush et al., 1998). *Aphanomyces* spp. are also responsible for causing EIDs, for example, Epizootic Ulcerative Syndrome (EUS) commonly known as red spot in over a hundred freshwater and estuarine fish species worldwide (Chinabut et al., 1995; Lilley et al., 1997; Boys et al., 2012). EUS has been recognized in Australia (Huchzermeyer and Van der Waal, 2012) and the Philippines (Callinan et al., 1995) since 1972 and 1995 respectively, however in 2006 this fungal pathogen was sighted in the Zambezi River System (ZRS), Africa, the pathogen had travelled further along the ZRS inhabiting several new ecosystems (Huchzermeyer and Van der Waal, 2012; Nsonga et al., 2013). Additionally, *Aphanomcyes* spp. low host specificity increases its prevalence among a range of species, increasing disease outbreaks in the ZRS, which is home to approximately eighty species and thus becoming a great concern in disease control (Huchzermeyer and Van der Waal, 2012).

However, compared to farmed fish populations, monitoring EIDs in wild populations can prove difficult as fish are constantly moving long distances beneath turbid waters, which means they can go undetected and underreported distorting our understanding of the effects on these populations (Gozlan, 2012). Globally, EIDs have caused high mortalities in farmed populations (Torto-Alalibo et al., 2005; Phillips et al., 2008; Van Den Berg et al., 2013). This is important information for wild populations because there are several instances where transmission of fungal and fungal-like microbes can occur between the two environments. For example, farmed fisheries often drain into rivers (Andreou et al., 2012) allowing the transfer of microbes and other organisms (Krkosek et al., 2005; Hilborn, 2006). *Pseudorasbora parva* (topmouth gudgeon), widely known by its aquaculture and ornamental fish trade, is a healthy carrier of *S. destruens* (Gozlan et al., 2005). Originally and unintentionally imported from China, the topmouth gudgeon's propitious nature has allowed it to become a profound invader in wild environments, invading thirty five new countries over the past 40 years, where for example *S. destruens* was identified in river systems of the Netherlands and the UK, posing great threats to native fish populations (Gozlan et al., 2010; Spikmans et al., 2013).

Previous studies have shown that susceptibility of farmed fish to fungal or fungal-like microbes depends on several factors including rapid drops in ambient temperatures (Bly et al., 1993; Lategan et al., 2004), low water levels, failure to remove dead fish or eggs, primary infection by other organisms, (Piper et al., 1982; Plumb, 1984) and pollution (Wu et al., 2010), all of which can reduce ecosystem function (Chapin et al., 2000; Cowx and Gerdeaux, 2004; Peeler et al., 2010) and lead to an increase in EIDs (Woolhouse and Gowtage-Sequeria, 2005). Thus, we could expect that such environmental drivers at play in the wild would potentially have also a direct impact on the emergence of fungal pathogens. In particular, the recent paper by Vörösmarty et al. (2010) shows that 65% of rivers worldwide regarding thermal and water level disturbances are under moderate-high threat, in particular Asia and North America. However, Vörösmarty et al. (2010) goes on further to highlight the fact that there is a lack of knowledge and investment being directed to biodiversity conservation, with an increase in EIDs, species distinctions, human population, climate change and habitat destruction (Vörösmarty et al., 2010; Huchzermeyer and Van der Waal, 2012; Nsonga et al., 2013). It will be important to monitor these river systems and to reduce these pressures, consequently allowing the populations, species distributions and diversity of fish to remain sustainable for the future.

PERSPECTIVES AND CONCLUSIONS

Since, the initial discovery of the fungal chytrid pathogen 20 years ago (Berger et al., 1998), several studies have reported its

Table 2 | Example of fungal infections in wild fish population.

Host (Family)	Pathogen	Location	Prevalence	Mortality	References
SALMONIDS					
Oncorhynchus tshawytscha (Chinook Salmon) *Oncorhynchus mykiss* (Rainbow Trout)	*Saprolegnia parasitica*	Columbian & Snake Rivers, United States of America.	–	22%	Neitzel et al., 2004
Salmo salar (Atlantic Salmon) *Salmo trutta* (Sea Trout)	*Saprolegnia diclina*	River North Esk, Scotland.	30%	–	Roberts et al., 1972
Oncorhynchus tshawytscha	*Sphaerothecum destruens*	Sacramento River, United States of America.	32%	–	Arkush et al., 1998
CLUPEIDS					
Clupea harengus (Bony Herring)	*Ichthyophonus hoferi*	Skagerrak-Kattegat Area, Sweden.	1.1%	8.9%	Rahimian and Thulin, 1996
CHARACIDS					
Astyanax eigenmanniorum *Astyanax fasciatus*	*Saprolegnia parasitica*	Central Argentina.	95%	–	Mancini et al., 2008
CICHLIDS					
Sargochromis giardia (Pink Bream) *Brycinus lateralis* (Stripped Robber)	*Aphanomyces invadans* (EUS)	Zambezi River System, Africa.	3–37.5%	–	Huchzermeyer and Van der Waal, 2012; Nsonga et al., 2013
CYPRINIDS					
Pseudorasbora parva (Topmouth Gudgeon)	*Sphaerothecum destruens*	Meuse River, Netherlands.	67–74%	–	Spikmans et al., 2013
Leucaspius delineates (Belica)	*Sphaerothecum destruens*	Stoneham Lakes system, United Kingdom.	5%	–	Andreou et al., 2011
PERCIDIDS					
Leiopotherapon unicolor (Spangled Perch) *Macquaria ambigua* (Golden Perch)	*Aphanomyces invadans* (EUS)	Murray-Darling River System, Australia.	10% (2008) 29% (2010)	–	Boys et al., 2012

significant impact on amphibians along with major population declines worldwide (Skerratt et al., 2007). What is interesting with this particular pathogen is the relatively good epidemiological data, which have allowed the progression of the disease to be tracked on a global scale and in many wild amphibian populations.

However, there is currently not enough epidemiological data related to fungal pathogens of fish. In light of the recent emergence of *S. destruens*, which poses a threat to European fish diversity (Gozlan et al., 2005; Andreou et al., 2012), it is likely that patterns of ecological impacts similar to those found in chytrid parasites of amphibians, are at play in freshwater fish populations (Gozlan, 2012). For example, as the great majority of *S. destruens* cases are driven by the invasion of a healthy fish carrier, it is expected, as shown by Spikmans et al. (2013), that additional monitoring of invaded wild fish communities would show the presence of this fungal-like infectious pathogen. In fact, there is not enough fungal pathogen data mostly for fish from wild populations. It is interesting to note that the dominant reporting of fish fungal and fungal-like pathogens has come from the aquaculture sector with very limited reports on fungal emergence in wild fish populations (see **Table 2**). The key reason is likely to be a combination of a lack of external pathological specificity of infected hosts and the chronic nature of some of the diseases caused by these fungal and fungal-like pathogens, which, in contrast to viral pathogens, spread over longer periods of time.

Research should be modeled after the amphibian chytrid research structure with a lot more systematic tracking of these pathogens. For example, the recent paper by McMahon et al. (2013) clearly indicates that even for the chytrid, which has been well studied in the wild, new potential non-amphibian hosts could contribute further to its dispersal, prevalence and virulence. Similarly, it would thus be pertinent to determine if fungal and fungal-like pathogens of fish that have a high generalist index could include non-fish hosts and thus contribute to a wider dissemination of some fungal related diseases beyond the immediate local fish communities. Additional monitoring of wild fishes is also needed. Identification and surveys of environmental drivers of fungal pathogens would improve understanding of the ecological risks of disease emergence in aquatic communities (Copp et al., 2009, 2010). This should also be the concern of the aquaculture sector, as strong pathways exist between wild and farmed fish with truly biosecure fish farms being the exception. The pan-extinction of sunbleak *Leucaspius delineatus* populations in Europe in less than 40 years should be a reminder of the risk associated with an un-controlled epizooty of fungal pathogens

(see Gozlan et al., 2005, 2010). Environmental surveys to identify ecological drivers of fungal pathogens in fishes are also key in characterizing the underpinning drivers of fungal and fungal-like pathogen emergence. For example, fish fungal pathogen emergence such as the EUS in Africa could well be linked to current environmental changes occurring in African rivers (Vörösmarty et al., 2010). Thus, in light of the importance of freshwater fish for millions of people around the world, particularly in developing countries, in addition to biodiversity conservation perspectives, pathologists should make a concerted effort to increase their monitoring of fungal pathogens in wild fish populations. Currently, PCR is the method that should be used for monitoring. Along with an increasing reduction in PCR associated cost, their sensitivity and specificity should facilitate such regular monitoring of the wild fish compartment.

In conclusion, our review of fungal and fungal-like pathogens of fish has highlighted current knowledge gaps that need to be rapidly filled if future epizootics are to be prevented. It has also indicated that epidemiological elements arising from other non-fish specific fungal pathogens could be used to refine our true understanding of current and future ecological impacts of these types of pathogens on global fish diversity. For example, existing experimental data arising from fungal pathogen challenges of fish should be used to develop SEIR models (i.e., susceptible-exposed-infectious-recovered) specific to fungal pathogens and fish hosts. This would allow a simulation of the true extent of the ecological risk and provide elements for a better environmental monitoring and understanding of these types of pathogens.

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Review

Ecology of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in temperate agroecosystems: Potential for conservation biological control

Nicolai V. Meyling *, Jørgen Eilenberg

Department of Ecology, Faculty of Life Sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark

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Abstract

It is increasingly recognized that the biodiversity in agroecosystems deliver significant ecosystem services to agricultural production such as biological control of pests. Entomopathogenic fungi, specifically the anamorphic taxa *Beauveria bassiana* and *Metarhizium anisopliae*, Hypocreales (Ascomycota), are among the natural enemies of pests in agroecosystems and the fungi are candidates for future conservation biological control in temperate regions. Conservation biological control is a biological control strategy in which farming practices and environmental manipulations are adopted to enhance the living conditions for specific natural enemies of pests. However, in order to manipulate the environment for the benefit of populations of the entomopathogens, knowledge of fundamental aspects of the ecology of the fungi considered is necessary. This knowledge is still scarce despite the large bulk of recent research into inoculation and inundation biological control with these fungi. Here, we review the current knowledge of the ecology of indigenous populations of *B. bassiana* and *M. anisopliae* in agroecosystems of temperate regions, primarily Europe and North America. We suggest anamorphic life cycles of *B. bassiana* and *M. anisopliae* in these regions based on the literature of their natural occurrence and distribution in agroecosystems, population dynamics, and interactions with other organisms, environmental factors, and agronomical practices.

Keywords: Ecosystem services; Field margins; Functional biodiversity; Pest control; Population dynamics; Natural enemies

1. Introduction

Studies of biodiversity in agroecosystems and the delivery of ecosystem services to agricultural production have usually ignored the contribution of entomopathogens in the regulation of pest populations (Altieri, 1999; Gurr et al., 2003; Tscharntke et al., 2005). However, entomopathogens are among the natural enemies of arthropod pests in agroecosystems. An improved understanding of the ecology of indigenous populations of these beneficial organisms is a prerequisite for the evaluation of their contributions to pest control and for predicting the impact of agricultural practices on their populations.

The anamorphic entomopathogenic fungi *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metschnikoff) Sorokin from the order Hypocreales (Ascomycota) are natural enemies of a wide range of insects and arachnids and both fungi have a cosmopolitan distribution (Roberts and St. Leger, 2004; Rehner, 2005). Much effort has been put into research on the development of *B. bassiana* and *M. anisopliae* as biological control agents (for inundation and inoculation biological control) to be applied in agriculture and forestry in temperate regions. However, this bulk of knowledge is in striking contrast to the lack of research into the fundamental ecology of these fungi in terrestrial ecosystems, including agroecosystems.

* Corresponding author.
E-mail address: nvm@life.ku.dk (N.V. Meyling).

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It is a future challenge for sustainable agricultural production to include pest control from entomopathogens in conservation biological control (referred to as CBC below) (Barbosa, 1998; Lacey et al., 2001). CBC is a biological control strategy in which farming management practices are adopted to enhance the living conditions for specific natural enemies of pests with the specific objective to suppress pest populations (Barbosa, 1998; Eilenberg et al., 2001). In recent years, an extensive number of studies have been published with focus on CBC with respect to natural enemies belonging to the arthropods, i.e., predators and parasitoids (e.g., Landis et al., 2000; Gurr et al., 2003; Kean et al., 2003; Chang and Snyder, 2004). So far, limited research effort has been devoted to the effects of environmental manipulations on entomopathogens (Fuxa, 1998; Ekesi et al., 2005). Emphasis has been given to aphid pathogenic fungi from Entomophthorales: *Pandora neoaphidis* (Remaudière and Hennebert) Humber and its potential as a natural enemy in CBC was studied in the UK (Shah and Pell, 2003; Ekesi et al., 2005) and *Neozygites fresenii* (Nowakowski) Batko was studied in the US (Shah and Pell, 2003; Steinkraus, 2006). In both cases the authors concluded that these fungal pathogens showed high potential for CBC of aphids. However, insights into the fundamental ecology of the fungi in agroecosystems are essential to predict the effects of environmental manipulations on the fungal populations and thus to include entomopathogens in CBC (Fuxa, 1998; Lacey et al., 2001; Shah and Pell, 2003; Stuart et al., 2006). Information of pathogen persistence and dispersal in the environment is equally important (Fuxa, 1998). Knowledge of the ecology of indigenous populations of *B. bassiana* and *M. anisopliae* in agroecosystems in temperate regions as well as the effects of environmental conditions and agricultural practices on the fungi is therefore necessary if they are to be manipulated for CBC in the future. Here, we present the current knowledge of these ecological aspects with reference to *B. bassiana* and *M. anisopliae* in temperate agroecosystems.

We have based the review on the published literature on natural occurrence and fundamental ecology of the morphological species *B. bassiana* and *M. anisopliae*. Recent research has shown that the morphological species *B. bassiana* is paraphyletic (Rehner and Buckley, 2005) and consists of two separate clades that should be separated taxonomically. As this redescription has not yet been done, we use *B. bassiana* throughout this review as reference to the morphological species when no information of phylogenetic affiliation is available.

German studies have recently shown that the structure of the agricultural landscape has impact on agroecosystem biodiversity, which also comprises entomopathogenic fungi, and thus the ecosystem services they deliver (Tscharntke et al., 2005). We therefore include the aspect of landscape elements in this review. This review aims to create the foundations for future focus on the indigenous populations of *B. bassiana* and *M. anisopliae* as biological control agents in temperate regions using a CBC strategy.

2. Distribution and diversity of indigenous populations

2.1. Soil

The soil environment is usually the conventional isolation site for hypocrealean entomopathogenic fungi (Keller and Zimmerman, 1989; Hajek, 1997), and several species can be found in both cultivated and more natural habitats (Steenberg, 1995; Vänninen, 1996; Bidochka et al., 1998; Klingen et al., 2002; Keller et al., 2003; Meyling and Eilenberg, 2006b). In studies of natural occurrence of entomopathogenic fungi in the soil, susceptible bait insects such as *Galleria mellonella* L. (Lepidoptera: Pyralidae) or *Tenebrio molitor* L. (Coleoptera: Tenebrioidae) are usually added to soil samples in order to recover fungal isolates; in principle the method can be described as the use of selective media.

Keller et al. (2003) found *M. anisopliae* to be common in both arable fields and adjacent meadows, but the species occurred at higher densities in meadows. In Canada, *M. anisopliae* was most frequent in agricultural fields compared to forest habitats (Bidochka et al., 1998), while *M. anisopliae* in Finland was isolated more often in southern parts of the country and the occurrence was not adversely affected by cultivation of the soil (Vänninen, 1996). In Danish soils *M. anisopliae* was more frequent in sun exposed habitats (i.e., cultivated areas) than in shaded habitats (Steenberg, 1995), which is similar to the Canadian findings. Furthermore, *M. anisopliae* was not among the entomopathogenic fungi isolated in the soil of a Danish forest ecosystem (Nielsen et al., 2004). This implies characteristics of *M. anisopliae* as an 'agricultural' species that is most common in exposed and regularly disturbed soil environments. Local and significant differences in natural distribution on one location can, however, occur. Meyling and Eilenberg (2006b) found *M. anisopliae* to be locally rare in a Danish agricultural field while *B. bassiana* was the dominant fungal species. Bidochka et al. (1998) found *B. bassiana* to be affiliated with shaded and uncultivated habitats (i.e., forests) and *B. bassiana* also occurred frequently in hedgerow soils at a Danish locality (Meyling and Eilenberg, 2006b).

These differences in natural occurrences in soil challenge the CBC strategy. Based on the studies made at a regional scale, indigenous populations of *M. anisopliae* appeared to be the most suitable candidates for environmental manipulations because *M. anisopliae* was most associated with agricultural field soil. However, in the case of the Danish agroecosystem described by Meyling and Eilenberg (2006b), *M. anisopliae* was found to be rare locally and *B. bassiana* would be a more suitable candidate for CBC. Thus the scale of the landscape needs to be considered when evaluating indigenous populations of entomopathogenic fungi in soils.

2.2. Insects

Entomopathogenic fungi occur naturally as infections in insect hosts which can be collected in the field and incubated in the laboratory for documentation of the fungus. *B. bassiana* have been documented to occur naturally in >700 species of hosts (Inglis et al., 2001). Studies on the prevalence of fungi in insects have usually been limited to species that are pests or are important non-target species such as certain predators and parasitoids. However, it is likely that almost any major insect taxon collected intensively will be found to be a natural host for *B. bassiana* in temperate regions. The occurrences of the fungi as infections in hosts are presumably the only part of the fungal life cycle in which the fungi can build up significant population sizes by producing vast numbers of conidia. Thus contributing to the availability of susceptible hosts for fungal population increase is a key component when considering environmental manipulations in CBC strategies.

2.3. Plant associations

Recent evidence suggests that both *B. bassiana* and *M. anisopliae* have the potential to engage in fungus–plant interactions. The large majority of investigated higher vascular plants have been found to host fungal endophytes (Saikkonen et al., 1998; Arnold and Lewis, 2005) including species in Clavicipitaceae contained within Hypocreales (White et al., 2002). *B. bassiana* has also been included in this spectrum of fungi with endophytic activity by infecting corn (*Zea mays*) (Bing and Lewis, 1991, 1992, 1993). Endophytic fungi are often regarded as plant-defending mutualists (Saikkonen et al., 2004) and the presence of *B. bassiana* in internal plant tissue has been discussed as an adaptive protection against herbivorous insects (Elliot et al., 2000; White et al., 2002). Besides natural occurrence in leaf tissue of corn, *B. bassiana* exhibited endophytic activity in cacao (*Theobroma cacao*) (Posada and Vega, 2005), poppy (*Papaver somniferum*) (Quesada-Moraga et al., 2006) and coffee (*Coffea* spp.) and tomato (*Lycopersicon esculentum*) (F.E. Vega, personal communication). In temperate regions, inoculum of *B. bassiana* has furthermore been isolated from phylloplanes of various plants in hedgerows in Denmark (Meyling and Eilenberg, 2006a). This occurrence was hypothesized to be a consequence of deposition from the surroundings but was also suggested to act as a natural infection pathway of endophytic activity (Meyling and Eilenberg, 2006a). These new findings open exciting perspectives for the understanding of the ecology of *B. bassiana*. However, no knowledge is currently available about natural endophytic activity and host plant range of *B. bassiana* in temperate regions or of the significance of *B. bassiana* as an endophyte for fungus or plant fitness.

Plant association was also recently documented for *M. anisopliae*, but this association occurred below ground in the rhizosphere (Hu and St. Leger, 2002). The rhizosphere is the layer of soil immediately surrounding the root and many interactions between plants and other organisms occur in this interface (Bais et al., 2006). By releasing a recombinant isolate of *M. anisopliae* to the soil of an experimental cabbage field in MD, USA, Hu and St. Leger (2002) were able to demonstrate that the released isolate persisted better in the soil immediately surrounding the cabbage roots as compared to the bulk soil. Factors in the rhizosphere therefore seemed to promote the persistence and biological activity of *M. anisopliae* (Hu and St. Leger, 2002). Wang et al. (2005) further documented that *M. anisopliae* expressed similar genes when growing in exudates from bean roots and on a nutrient rich medium while different genes were expressed by the fungus when growing on insect cuticle and in insect hemolymph. This indicated that *M. anisopliae* has developed different adaptations to function as a pathogen and to grow saprophytically in the rhizosphere (Wang et al., 2005). The implication for biological control with *M. anisopliae* exploring the rhizosphere competence was investigated by Bruck (2005). Inoculated conidia of *M. anisopliae* persisted significantly better (up to one year) in the rhizosphere of *Picea abies* compared to the bulk soil (Bruck, 2005). Survival outside the host may thus be critical for the ability of *M. anisopliae* to control insect pests in the soil (Roberts and St. Leger, 2004; Bruck, 2005). Whether the rhizosphere of plants generally provide a ''refuge'' (where the fungus can survive outside insect hosts) for *M. anisopliae* in the soil remains to be investigated. Perhaps associations with plants are important in the life cycle of both *B. bassiana* and *M. anisopliae* in temperate regions.

The compartments in temperate terrestrial ecosystems in which *B. bassiana* and *M. anisopliae* occur naturally are summarized in Fig. 1. Compartments are connected with lines to indicate dispersal and infection pathways when these are known; these aspects are presented below. Plant associations are included as potential compartments for the natural occurrence of *B. bassiana* and *M. anisopliae*.

2.4. Genetic diversity in populations of *B. bassiana* and *M. anisopliae*

Biodiversity is usually evaluated by assessment of species diversity. Biodiversity assessment of fungal communities is, however, challenging, because fungal taxa often consist of complexes of cryptic species (Bickford et al., 2007). Cryptic species are found in *B. bassiana* (Rehner and Buckley, 2005; Rehner et al., 2006) and seemingly also in the morphological species *M. anisopliae* (Bidochka et al., 2001, 2005). Evaluating the biodiversity contribution of *B. bassiana* and *M. anisopliae* in agroecosystems must therefore be based on an assessment of the genetic diversity to discover potential cryptic species. Traditional assessment of fungal species diversity is based on morphological features. Unfortunately, few and sometimes ambiguous characters are used for species separation. Furthermore, many entomopathogenic hypocrealean fungi have probably exclusively anamorphic life cycles in temperate regions, at

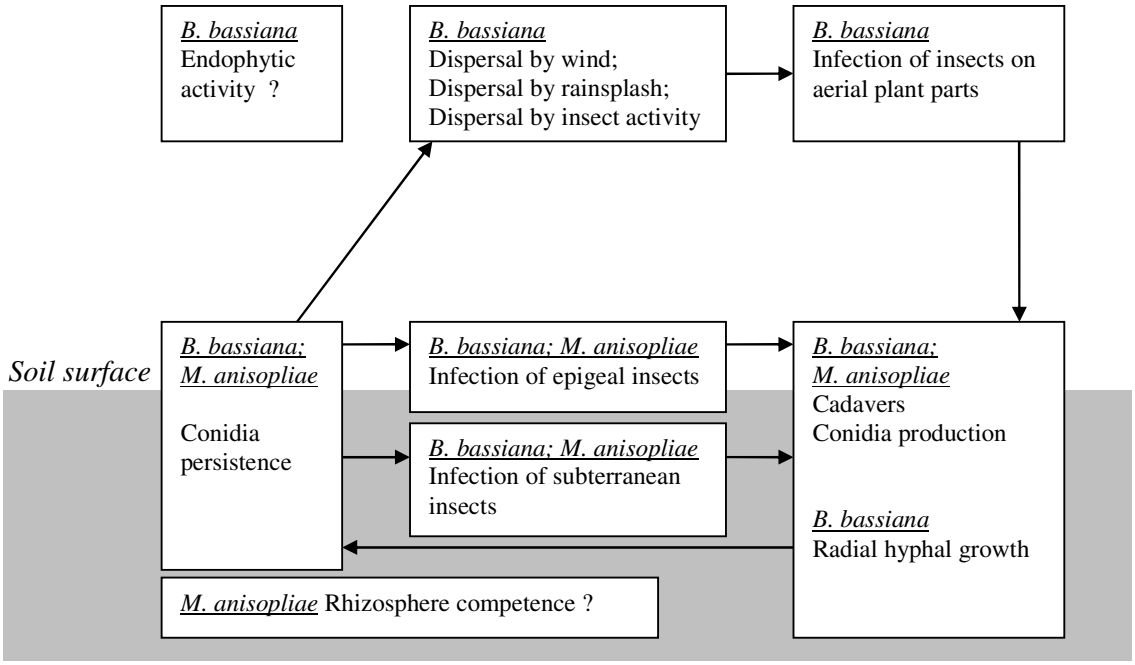

Fig. 1. Suggestions for anamorphic life cycles of *Beauveria bassiana* and *Metarhizium anisopliae* in Northern temperate regions. The grey area represents the soil environment while the white background of the figure is above ground. The life cycles are based on the current knowledge of the ecology of the fungi in temperate regions as presented in the text. Significant dispersal and infection pathways are indicated by arrows. In each ecological compartment, fungus names indicate the significance of the compartment for the life cycles of *B. bassiana* and *M. anisopliae*. Compartments representing plant associations are included with no arrows to indicate the possible significance of these associations, but the unknown relationships with other compartments.

least outside East Asia, which additionally complicates matters about defining biological species and comparing intraspecific and interspecific genetic diversity. Recent advances in molecular techniques have shed new light on our understanding of species boundaries, especially within the genus *Beauveria*. Several studies have revealed much genetic diversity of the morphological species *B. bassiana* (Glare, 2004). However, a recent phylogeny of *Beauveria* spp. showed that the morphological species *B. bassiana* in fact is paraphyletic and consists of two unrelated clades of which one is more related to *Beauveria brongniartii* (Saccardo) Petch than to the second "*B. bassiana*"-group (Rehner and Buckley, 2005). This latter group is tentatively referred to as 'pseudobassiana' (Rehner et al., 2006) but it needs a formal description. The existence of two unrelated clades may explain some of the large genetic diversity reported in the morphological species *B. bassiana*. Furthermore, the two groups of *B. bassiana* are themselves assemblages of cryptic species or separate clades (Rehner and Buckley, 2005; Rehner et al., 2006) thus each group also contains much genetic diversity. Both groups infect a wide range of insects and can be isolated from the soil (Meyling, 2005; Rehner and Buckley, 2005) but unfortunately no data currently exists on differences in ecological niches between the groups. As mentioned above, *B. bassiana* is used here to refer to the morphological species when no other information is known.

Most studies of genetic diversity of *B. bassiana* have been based on isolates from culture collections and have usually been compiled to present isolates from infections in certain pest insects or isolates originating from a (major) geographical region. However, to understand the ecology of the indigenous fungal populations, studies must be carried out on isolates collected at a local scale and in different spatial compartments of the ecosystem. These isolates will represent genotypes that potentially interact with host populations, with each other and the environment under field conditions.

Based on the latter sampling strategy, *B. bassiana* isolates were sampled within the boundaries of a single organic agroecosystem in Denmark with the specific objective to characterize the genetic diversity. Sequence analyses [genomic regions as described in Rehner and Buckley (2005) and Rehner et al. (2006)] showed that several phylogenetic lineages were present in this single agroecosystem (Meyling, 2005; Meyling et al., 2005). Table 1 shows that the Danish locality contained the morphologically similar groups *B. bassiana sensu lato* and 'pseudobassiana'. Furthermore, isolates from each individual clade of *B. bassiana s.l.* were more related to isolates from other European countries than they were to isolates from other clades at the locality. Only one of these clades was represented among isolates from the arable field soil (Table 1). At the same site, the morphological species *B. bassiana* was the most frequent entomopathogenic fungus in the soil of the agricultural field (Meyling and Eilenberg, 2006b). This study demonstrated that different genetic groups of the morphological species *B. bassiana* coexisted at a local scale

Table 1
Genetic groups of isolates of the morphological species *B. bassiana* collected within a single agroecosystem (an organic field and hedgerow) at Bakkegården, Denmark

Monophyletic group	Habitat of isolates within locality	Country of origin of most related isolate
B. bassiana s.l.[a]		
Eu_1	Arable field; Hedgerow	Hungary, ARSEF 1628
Eu_3	Hedgerow	France, ARSEF 1185
Eu_4	Hedgerow	Belgium, ARSEF 1848
Eu_5	Hedgerow	NA
Eu_6	Hedgerow	France, ARSEF 815
'pseudobassiana'[b]	Hedgerow	

Groups were identified based on DNA sequences of genomic regions as described by Rehner and Buckley (2005) and Rehner et al. (2006). Isolation habitats (i.e., arable field or hedgerow) for isolates belonging to each monophyletic group are presented. Isolates from reference collections that were found to belong to similar monophyletic groups are also presented. Results are summarized from Meyling (2005).

[a] Monophyletic clade of the morphological species *B. bassiana* refered to as "Clade A" by Rehner and Buckley (2005) and *B. bassiana s.l.* by Rehner et al. (2006).

[b] Monophyletic clade of the morphological species *B. bassiana* refered to as "Clade C" by Rehner and Buckley (2005) and 'pseudobassiana' by Rehner et al. (2006).

in the agricultural landscape. The study further indicated that typical North European agroecosystems bordered by hedgerows may harbor significant sources of genetic diversity of entomopathogenic fungi. However, the agricultural field soil only harbored one of the identified *B. bassiana s.l.* clades, documenting the lack of biodiversity in a monocultural cropping system, even when organic.

Very few studies have been carried out on the genetic diversity of *M. anisopliae* at a local or regional scale. Most knowledge has been generated in Canada on *M. anisopliae* isolates from different habitats, i.e., agricultural and forest sites. Bidochka et al. (2001) demonstrated that *M. anisopliae* isolates from agricultural soils belonged to a specific genetic group distinguished from isolates originating from forest soils. These two groups of *M. anisopliae* could be considered as separate cryptic species (Bidochka et al., 2001, 2005). However, there is currently very limited knowledge about the composition and distribution of *M. anisopliae* populations at a local scale. Hu and St. Leger (2002) described selected indigenous isolates of *M. anisopliae* from a local cabbage field in MD, USA, as belonging to two separate explicit allozyme groups. However, specific genetic studies of local populations of *M. anisopliae* still need to be conducted.

The Canadian studies (Bidochka et al., 2001) on *M. anisopliae* further generated the interesting conclusion that isolates belonging to the genetic group in agricultural habitats exhibited heat-tolerance for *in vitro* growth as well as stronger UV-light resistance as compared to the group from forested habitats (Bidochka et al., 2001). Thus Bidochka et al. (2001) proposed the hypothesis that the abiotic conditions (i.e., UV-radiation, exposure to elevated temperatures) in the habitat selected for the genetic group of *M. anisopliae* that could survive in that given habitat. This hypothesis suggested that the time-window experienced outside the host was the most important factor for pathogen survival in the environment. Furthermore, it was indicated that similar characteristics could be found among *B. bassiana* isolates collected in Canada (Bidochka et al., 2002). This corroborates with the distribution of genetic groups of *B. bassiana s.l.* in the Danish agroecosystem summarized in Table 1. Here, only one group could be found in the agricultural soil while several genetic groups were harbored in the soil of the neighboring hedgerow (Meyling, 2005; Meyling et al., 2005). Obviously, more studies are needed to verify if this is a general trend in agricultural landscapes in temperate regions and whether the different genetic groups of *B. bassiana s.l.* exhibit variation in their realized niches under different abiotic conditions. If only specific genetic groups of the fungi can survive in agricultural fields then this will be a challenge for environmental manipulations in a CBC strategy targeting *B. bassiana* and *M. anisopliae*.

3. Population dynamics

3.1. Population increase and infections of hosts

Entomopathogenic fungi rely on arthropod hosts to build up population levels of infective stages (mitosporic conidia). During the cropping season outbreaks of diseases can regularly be observed in insect populations in the field, referred to as epizootics. Generally, the development of epizootics rely on host population dynamics, the number of infective stages in the pathogen population and the viability of these, infection efficiency and development (Anderson and May, 1981) and a complex set of environmental factors and timing (Inglis et al., 2001). Considerable information on the biology of the organisms as well as specific environmental parameters (in time and space) is necessary to understand and predict the development of epizootics. Key components of population dynamics of the entomopathogenic fungi are the build up of the population, the infection of hosts, and the survival and dispersal in the environment (Anderson and May, 1981).

Both *B. bassiana* and *M. anisopliae* need resources from a host individual to grow and build up fungal biomass. It is conventional to consider the hypocrealean entomopathogenic fungi as saprophytic because many species grow well on artificial media. However, the status of *Metarhizium* spp. as true saprophytes is not well established (Roberts and St. Leger, 2004). Moreover, *Beauveria* spp. and *Metarhizium* spp. are poor competitors for organic resources compared to opportunistic saprophytic fungi that are ubiquitous in soils (Keller and Zimmerman, 1989; Hajek, 1997). It seems more likely that the fungi lurk as conidia in the soil environment waiting to infect a new host. However, *M. anisopliae* may be activated by root exudates in the rhizosphere as described above. Despite this new evidence, insect hosts should still be considered to constitute the principal

source of organic matter for fungus population build-up. The host is the "home ground" of the entomopathogenic fungi, and opportunistic microorganisms are assumed to be held effectively at bay by antimicrobial substances produced by the fungus (Boucias and Pendland, 1998). However, there is very scant published evidence for the antimicrobial activities of the compounds released by these fungi and accordingly Boucias and Pendland (1998) did not provide any references for their claim. A recent documentation of antimicrobial activity of substances from *B. bassiana* (Oller-López et al., 2005) indicates that the assumption is plausible. Field collected living insects, which are incubated in the laboratory, are regularly found infected by *B. bassiana,* yet limited published quantitative data exists on the prevalence of mycosed cadavers in the field. In Denmark, careful inspection of the litter layer beneath stinging nettles (*Urtica dioica*) in a hedgerow revealed that dead insects sporulating with *B. bassiana* were relatively abundant among leaf litter and soil: densities of 3.5–8.8 cadavers per m^2 could be recovered in July in separate patches (N.V. Meyling, unpublished data). Although these data were preliminary they indicated that cadavers were common on the soil surface, probably mostly in hedges and under natural vegetation. Furthermore, the results indicated that population levels of *B. bassiana* were boosted during the season by conidia production from mycosed insects in the litter layer beneath their host plants.

Conidia are produced in a single event which is consistent with the definition of semelparous reproduction (Pringle and Taylor, 2002; Hughes et al., 2004). Vast numbers of conidia are produced from a single infected insect cadaver (Gottwald and Tedders, 1982). The production of conidia is a key fitness parameter in asexual semelparous filamentous fungi (Pringle and Taylor, 2002) and has been used as a measure of cost in intraspecific competition in *M. anisopliae* (Hughes et al., 2004). However, the persistence and the effectiveness of infection of conidia are also critical properties for the ecological fitness of entomopathogenic fungi. Most conidia are likely to disintegrate quickly in the environment and only minimal proportions will presumably succeed in infecting new hosts. Infection success is density dependent and the number of conidia must exceed a critical threshold level or minimal viable population size (Hughes et al., 2004). Thus the acquisition of the threshold of infective conidia by a susceptible host is necessary for the continued survival of the fungus.

3.2. Dispersal in the environment

Dispersal of infective stages of a pathogen is an important factor in disease development (Anderson and May, 1981). Infective propagules of entomopathogenic fungi in the Hypocreales are passively dispersed, and this is mainly considered to occur through the action of weather components like wind and rain (Hajek, 1997; Inglis et al., 2001; Shah and Pell, 2003). In air samples, *B. bassiana* was isolated among a large array of airborne fungi (Airaudi and Marchisio, 1996; Shimazu et al., 2002; Ulevicius et al., 2004) and deposition from the air could be one likely source of the newly documented occurrence of *B. bassiana* on phylloplanes of hedgerow plants (Meyling and Eilenberg, 2006a). However, localized transmission onto plant parts by rain splash has also been shown (Bruck and Lewis, 2002b) but rainfall also removed fungus inoculum that had been applied to foliage (Inglis et al., 1993, 1995, 2000, 2001). In the soil environment the hypocrealen entomopathogenic fungi can persist, but extensive proliferation and dispersal are limited. Population build up relies on the conversion of host cadaver resources into infective conidia that are released from cadavers over time following sporulation (Gottwald and Tedders, 1982). The number of conidia released per host is dependent both on fungus species, host species, and host size. For example, *B. bassiana* released 10–200 times more conidia than *M. anisopliae* from adult pecan weevils (Gottwald and Tedders, 1982). Additionally, *B. bassiana* radiated out from weevil cadavers in the soil by hyphal growth and subsequently infected larvae in neighbouring experimental cells while *M. anisopliae* growth was restricted to the surface of the cadaver (Gottwald and Tedders, 1984).

Entomopathogenic fungi are dispersed by living infected hosts which migrate and die in another place than where they became infected (Hajek, 1997). Several aphid species migrate long distances high in the atmosphere and migrating aphids were found to harbour several entomopathogenic fungi (Entomophthorales and *B. bassiana*) (Feng et al., 2004). This implies that *B. bassiana* is able to travel over long distances as infections in hosts, which can later lead to new infections and establishment far away from the original site of the fungus.

The potential of arthropods to disperse and vector entomopathogenic fungi by their activity has been demonstrated in different terrestrial ecosystems. In the soil, collembolans dispersed conidia of *B. bassiana* and *M. anisopliae* which were not pathogenic to them (Dromph and Vestergaard, 2002), both by carrying conidia on the cuticle and by ingesting conidia which, after passage through the digestive tract, could remain viable (Dromph, 2001). Moreover, collembolans were able to vector inoculum to other soil-dwelling insects and initiate infections in laboratory experiments (Dromph, 2003). Also soil-dwelling mites were shown to be potential vectors of *B. bassiana* (Renker et al., 2005).

Insects inhabiting nettle plants were able to disperse inoculum of *B. bassiana* by their activity (Meyling et al., 2006) and could therefore be the distributors of naturally occurring *B. bassiana* on nettle phylloplanes as an alternative to wind dispersal (Meyling and Eilenberg, 2006a; Meyling et al., 2006). Moreover, predators initiated infections in aphids after vectoring inoculum from sporulating cadavers in the nettle canopy (Meyling et al., 2006). Interspecific vectoring of *B. bassiana* has additionally been shown in corn systems where fungivorous beetles dispersed inoculum to larvae of the European corn borer *Ostrinia nubialis*

(Lepidoptera: Pyralidae) in the tunnels of the latter (Bruck and Lewis, 2002a). Insect activity may therefore influence the dispersal of conidia of *B. bassiana* as indicated on Fig. 1. Thus CBC strategies that aim to manipulate the dispersal of insects may also affect dispersal of entomopathogenic fungi in the agroecosystem.

4. Effects of agricultural practices and structure of the agroecosystem

4.1. Soil disturbance and environmental factors

Annually cropped agroecosystems are highly disturbed mostly due to tillage regimes and this affects the populations of natural enemies of crop pests. The communities of entomopathogenic fungi in the arable soil environments are different from communities of less disturbed habitats (Steenberg, 1995; Bidochka et al., 1998; Meyling and Eilenberg, 2006b) and less disturbance in the cropping system also affect the populations of the fungi. In corn fields in the US, soil densities of *B. bassiana* (as measured by colony forming units per g of soil) under different tillage regimes were very variable between years, but were seemingly higher in no-tillage systems compared to systems subjected to ploughing and chiselling (Bing and Lewis, 1993). Likewise, conservation tillage regimes, using strip-till and no-till, were more favorable to *B. bassiana* and *M. ansiopliae* populations in the soil than conventional tillage regimes employing ploughing and disking (Hummel et al., 2002a). Furthermore, no-till cultivation in soybean and wheat positively affected the population levels of *B. bassiana* and *M. anisopliae* compared to conventional tillage (Sosa-Gomez and Moscardi, 1994). These findings of higher fungal densities in reduced tillage and no-till systems could be observations of indirect effects caused by increased levels of host populations of non-pest insects. High population levels of non-pest insects have been observed in reduced tillage systems (Hummel et al., 2002b). The observations cited above may therefore not necessarily be a direct result of mechanical disturbance on fungal population levels.

Exposed fungal inoculum is usually inactivated by the UV-components of solar radiation (Fargues et al., 1996, 1997b). Other abiotic factors affecting entomopathogenic fungi include temperature (Inglis et al., 2001) with strains exhibiting different temperature optima for growth (Fargues et al., 1997a). Indeed, temperature, moisture and UV-radiation seem to be most important for *B. bassiana* survival (Meikle et al., 2003). Persistence of applied fungus material in soils has been studied for several isolates of different species but the complexity of the soil environment makes it difficult to evaluate single factors determining survival (Inglis et al., 2001). Factors such as soil texture (Groden and Lockwood, 1991), pH values and moisture contents (Lingg and Donaldson, 1981) have been explored and are thoroughly reviewed by Inglis et al. (2001) and Klingen and Haukeland (2006).

Recent evidence suggests that certain genotypes of *M. anisopliae* and *B. bassiana* are dominating in soils of agroecosystems in Canada (Bidochka et al., 2001, 2002) and this also seems to apply to Danish conditions for *B. bassiana* (Meyling, 2005; Table 1). The reason for this observation could be adaptations of specific genetic groups to be more resistant to UV-radiation and temporal elevated temperatures. UV resistance and growth tolerance at high temperatures were characteristics of the isolates belonging to the genetic groups from agricultural soil in Canada (Bidochka et al., 2001, 2002). Given that these observations apply to populations of *B. bassiana* and *M. anisopliae* in general, other genotypes of these fungi could occur in the cropping system by the provisioning of sheltered and non-tilled habitats close to the crop in a CBC strategy.

4.2. Use of agrochemicals

Chemical insecticides, herbicides and fungicides are usually applied in conventional farming practices. These compounds, especially fungicides applied against plant pathogens, might also negatively affect the populations of entomopathogenic fungi with reduced pest regulation potential as a consequence.

Klingen and Haukeland (2006) provided a detailed review of published studies of effects of chemical pesticides on entomopathogenic fungi and nematodes. Their main conclusions were that insecticides and herbicides were not very harmful to fungal growth while fungicides were sometimes harmful (Klingen and Haukeland, 2006). However, most studies were performed *in vitro* with fungal cultures and extrapolation from studies in laboratory experiments to field conditions may not be straightforward. In the UK, for example, previous field application of the fungicide benomyl correlated with a lower incidence of *B. bassiana* in soil samples (Mietkiewski et al., 1997; Chandler et al., 1998). *In vitro* experiments further showed that the fungicide triadimefon inhibited the growth of *B. bassiana*, but fields previously treated with this product showed a higher frequency of occurrence of the fungus in soil samples than in samples from untreated control soils (Mietkiewski et al., 1997; Chandler et al., 1998). The fungicidal product albicarb even increased activity of *in vitro* cultures of *B. bassiana* (Mietkiewski et al., 1997). This emphasizes that due to the complex interactions and composition of agroecosystems applications of specific fungicides are not necessarily detrimental to the occurrence of entomopathogenic fungi in the soil. Selected compounds could thus possibly be used in integrated pest management (Mietkiewski et al., 1997).

4.3. Crop diversification

Mixtures of plants within the crop can reduce colonization by pest species and the use of trap crops can lure the pest insects away from the crop by a push–pull strategy (Hooks and Johnson, 2003; Cook et al., 2007). Manipula-

tion of insect behavior may also affect the dispersal of entomopathogenic fungi in agroecosystems because fungal inoculum can be distributed by insect activity.

Reducing the area of bare ground between the crop plants by mulching may reduce the population sizes of pests by enhancing conditions for ground dwelling predators (Hellqvist, 1996; Schmidt et al., 2004). Mulching may be unfavorable for hypocrealean entomopathogenic fungi as increased amounts of organic matter in soil have been shown to increase antagonistic activity against the fungi (Fargues and Robert, 1985; Studdert and Kaya, 1990).

Establishment of beetle banks within the fields for CBC targeted at populations of carabid beetles (Landis et al., 2000) could also promote populations of entomopathogenic fungi. Specifically, populations of certain genetic groups of *B. bassiana* or *M. anisopliae*, which have been documented to be absent from the cultivated soils in agroecosystems, could potentially benefit from beetle banks.

4.4. Importance of semi-natural habitats in agricultural landscapes

Semi-natural habitats (e.g., hedgerows) are important refuges for flora and fauna that do not thrive within the cultivated arable fields (Marshall and Moonen, 2002; Maudsley et al., 2002; Pywell et al., 2005). Striking differences in the communities of hypocrealean entomopathogenic fungi in soils between arable fields and hedgerows have been documented (Chandler et al., 1997; Meyling and Eilenberg, 2006b). It is likely that the populations of fungi are depending on the arthropod community in the hedgerows for their survival. Regarding *B. bassiana*, hedgerows constituted a tremendous reservoir of genetic diversity compared to agricultural soil (Table 1; Meyling, 2005). Permanent hedgerow habitats, preferably of some age, are valuable refuges of biodiversity in agricultural landscapes, including *B. bassiana* that could be manipulated for CBC.

Increasing evidence suggests that heterogeneity in the agricultural landscape is crucial for the maintenance of the diversity of species and ecological functional groups of organisms that are relevant for pest management in future sustainable agriculture (Benton et al., 2003; Weibull and Ostman, 2003). Landscape structure is also important when predicting the recruitment potential of organisms for biological control by changes in agricultural practices (Tscharntke et al., 2005). Both empirical evidence as well as simulation studies suggest that diversity in the guild of natural enemies is important for efficient biological pest control in agroecosystems (Wilby and Thomas, 2002; Cardinale et al., 2003). Thus initiatives for enhancing population levels of predators and parasitoids in CBC may simultaneously benefit the communities of entomopathogenic fungi in agroecosystems.

Thorough knowledge of the effect of semi-natural habitats on indigenous populations of entomopathogenic fungi is fundamental for their inclusion in CBC strategies (Shah and Pell, 2003). In the UK, studies have been carried out to include the entomophthoralean fungus *P. neoaphidis* in CBC of aphids. In this system, field margins constituted an important reservoir of fungus inoculum and predators acted as vectors of conidia among aphid populations (Roy et al., 2001; Ekesi et al., 2005). Nettles were particularly important reservoirs of *P. neoaphidis* because they harbored populations of nettle aphids that were susceptible to the fungus (Roy et al., 2001; Ekesi et al., 2005). In general, nettles in field margins also harbored many predators and parasitoids (Davis, 1973) and both phylloplanes and nettle specific insects harbored *B. bassiana* (Meyling and Eilenberg, 2006a; Meyling, 2005). Thus nettles (*U. dioica*) may be a key plant species for populations of predators, parasitoids and entomopathogenic fungi. Whether management strategies can facilitate the use of indigenous populations of *B. bassiana* in field margins in CBC remains to be elucidated.

5. Conclusion

Recent research has generated more knowledge of the natural occurrence, genetic diversity and dispersal mechanisms of *B. bassiana* and *M. anisopliae* in temperate agroecosystems. This knowledge is crucial to understand the ecology of entomopathogenic fungi and it is essential for the inclusion of the fungi in CBC. Molecular markers to assess genetic diversity are important tools in future studies of indigenous populations of *B. bassiana* and *M. anisopliae*. Initiatives to manipulate populations of predators and parasitoids in agroecosystems for CBC are likely to also benefit the populations of *B. bassiana* and *M. anisopliae*. However, there is a need for further understanding of the ecology of the entomopathogenic fungi in order to predict these effects.

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Adhesion of the Entomopathogenic Fungus *Beauveria* (*Cordyceps*) *bassiana* to Substrata†

Diane J. Holder and Nemat O. Keyhani*

Department of Microbiology and Cell Science, University of Florida, Gainesville, Florida 32611

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The entomopathogenic fungus *Beauveria bassiana* produces at least three distinct single-cell propagules, aerial conidia, vegetative cells termed blastospores, and submerged conidia, which can be isolated from agar plates, from rich broth liquid cultures, and under nutrient limitation conditions in submerged cultures, respectively. Fluorescently labeled fungal cells were used to quantify the kinetics of adhesion of these cell types to surfaces having various hydrophobic or hydrophilic properties. Aerial conidia adhered poorly to weakly polar surfaces and rapidly to both hydrophobic and hydrophilic surfaces but could be readily washed off the latter surfaces. In contrast, blastospores bound poorly to hydrophobic surfaces, forming small aggregates, bound rapidly to hydrophilic surfaces, and required a longer incubation time to bind to weakly polar surfaces than to hydrophilic surfaces. Submerged conidia displayed the broadest binding specificity, adhering to hydrophobic, weakly polar, and hydrophilic surfaces. The adhesion of the *B. bassiana* cell types also differed in sensitivity to glycosidase and protease treatments, pH, and addition of various carbohydrate competitors and detergents. The outer cell wall layer of aerial conidia contained sodium dodecyl sulfate-insoluble, trifluoroacetic acid-soluble proteins (presumably hydrophobins) that were not present on either blastospores or submerged conidia. The variations in the cell surface properties leading to the different adhesion qualities of *B. bassiana* aerial conidia, blastospores, and submerged conidia could lead to rational design decisions for improving the efficacy and possibly the specificity of entomopathogenic fungi for host targets.

Under intensive study for use as a biopesticide, the entomopathogenic fungus *Beauveria bassiana* displays a broad host range and is able to target a number of diverse arthropod species (7, 15, 16, 23, 26, 27, 31). Strains of *B. bassiana* have been selected for control of insects and other arthropods that act as disease vectors, including mosquitoes and ticks (6, 22); crop pests, such as whiteflies, caterpillars, grasshoppers, and borers (5, 9, 21, 25, 36); and even ecologically hazardous, invading pests, such as fire ants and termites (4, 8). The varied cuticles of these organisms represent the first barrier to the pathogen, and attachment of fungal propagules to the cuticle is the initial event in establishing mycosis. Air currents, dispersion via water droplets, and saprophytic growth over substrata inhabited by insects are considered the major routes for contact of fungal spores with host cuticles (2). Upon contact, fungal cells bind to the cuticle and initiate a developmental program that includes the production of specialized infection structures, such as germ tubes and penetrant hyphae (2, 17). If the infection is successful, the fungus grows across the cuticle surface and penetrates the host cuticle to invade and proliferate within the hemolymph, which ultimately results in the death of the host.

Fungal cell attachment to the cuticle may involve specific receptor-ligand and/or nonspecific hydrophobic and electrostatic mechanisms (2, 3, 10). A haploid anamorphic fungus, *B. bassiana*, produces a number of mononucleated single-cell types, including aerial conidia, blastospores, and submerged conidia, which can be isolated from agar plates, from rich broth submerged cultures, and from nutrient-limited submerged cultures, respectively. Although it is well known that culture conditions (and hence the cell type produced) can affect successful virulence for targeted hosts, little is known about the process of adhesion of *B. bassiana* cell types other than conidia. In this report we describe a quantitative assay used to determine the binding qualities and adhesion substratum preferences of *B. bassiana* aerial conidia, blastospores, and submerged conidia.

MATERIALS AND METHODS

Cultivation of fungi. *B. bassiana* ATCC 90517 was routinely grown on potato dextrose agar. Plates were incubated at 26°C for 10 to 14 days, and aerial conidia were harvested by flooding a plate with sterile distilled H_2O. Conidial suspensions were filtered through a single layer of Miracloth, and final spore concentrations were determined by direct counting using a hemocytometer. Blastospores were produced in Sabouraud dextrose–1 to 2% yeast extract liquid broth cultures using conidia harvested from plates at a final concentration of 0.5×10^5 to 5×10^5 conidia/ml as the inoculum. Cultures were grown for 3 to 4 days at 26°C with aeration. Cultures were filtered twice through glass wool to remove mycelia, and the concentration of blastospores was determined by direct counting. Submerged conidia were produced in TKI broth using fructose as the carbon source as described previously (32). For all cell types, Miracloth- or glass wool-filtered cell suspensions were harvested by centrifugation (10,000 × *g*, 15 min, 4°C), washed two times with sterile distilled H_2O, and resuspended to the desired concentration as indicated below (typically 10^7 to 10^8 cells/ml).

FITC labeling of *B. bassiana* cells. Fluorescein isothiocyanate (FITC) (100 μl of a 1-mg/ml stock solution per ml of fungal cells) was added to washed fungal cells (0.5×10^8 to 1×10^8 cells/ml) resuspended in 50 mM calcium carbonate buffer, pH 9.2. Each reaction mixture was incubated for 20 min in the dark, after which the cells were extensively washed (four or five times with an equal volume) with TB (50 mM Tris-HCl, pH 8.0). The final cell pellets were resuspended in TB to obtain the desired concentrations, as indicated below. The final cell suspension spore concentrations were checked by direct counting using a hemocytometer.

* Corresponding author. Mailing address: University of Florida, Microbiology and Cell Science, Bldg. 981, Museum Rd., Gainesville, FL 32611. Phone: (352) 392-2488. Fax: (352) 392-5922. E-mail: keyhani@ufl.edu.

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Adhesion assay. Two assays were used to assess adhesion to substrata. In the first (qualitative) assay, solutions of fungal cell suspensions (100 μl, 1×10^6 to 20×10^6 cells/ml) were placed in slide chambers (treated and untreated glass surfaces; Lab Tech chamber slide system; Nalgene Nunc, Naperville, IL) and incubated at 25°C and 100% humidity for various times. Adhesion was assessed microscopically after one wash or three washes. Digital images were taken using a Nikon Optiphot-2 microscope equipped with a digital camera. Adhesion was also assessed quantitatively using FITC-labeled cells incubated on various black microtiter plate test substrata. Fungal cell suspensions (100 μl, 1×10^6 to 20×10^6 cells/ml) were placed in (black) microtiter plate wells and incubated at 25°C in the dark for various times. Unbound cells were removed by aspiration of the liquid from the wells, followed by up to three washes with 450 μl TB. Fluorescence was measured using a Spectra Max Gemini XS microplate fluorometer (excitation wavelength, 495 nm; emission wavelength, 530 nm; cutoff wavelength, 515 nm; Molecular Devices Corp., Sunnydale, CA). For each experiment a standard curve of fluorescence intensity versus cell number (as measured by direct counting) was prepared. Typically, the fluorescence intensity was measured before washing (total number of cells) and after each wash. Weakly polar microtiter plates (Fluorotrac F200) and hydrophilic microtiter plates (Fluorotrac F600) were purchased from Greiner Biotech (Longwood, FL) and were used unmodified. Hydrophobic substrata were prepared by addition of a thin layer of silicone using Sigmacote (Sigma Corp., St. Louis, Mo.) to glass slides or to Fluorotrac F200 microtiter plate wells. Typically, substrata were treated up to three times with Sigmacote, and the treated plates or slides were placed in a fume hood overnight in order to ensure evaporation of all solvent.

Enzyme treatments. Aerial conidia, blastospores, and submerged conidia (0.5×10^7 to 1.0×10^7 cells/ml) were washed twice and resuspended in the enzyme reaction buffers suggested by the manufacturer. Portions (100 μl) of 10-mg/ml stock solutions of amylase (catalog no. A6255; Sigma), cellulase (catalog no. C9422; Sigma), or laminarinase (catalog no. L5272; Sigma) in 0.01 M KPO_4 (pH 6.8 for α-amylase and pH 5.6 for cellulase and laminarinase) were added to 0.9 ml of cells resuspended in the same buffer. For protease treatments, 100 μl and 50 μl of stock solutions (10 mg/ml and 1 mg/ml) of proteinase K (catalog no. p6911; Sigma) and pronase E (catalog no. 300140; Stratagene), respectively, in buffer (0.1 M Tris HCl, pH 7.8, 0.5% sodium dodecyl sulfate [SDS], 1 mM $CaCl_2$) were added to cells resuspended in the same buffer. Glycosidase reaction mixtures were incubated for 4 h at 25°C, and protease treatments were performed for 4 h at 37°C. After incubation, cells were extensively washed in 50 mM calcium bicarbonate buffer (pH 9.2) (seven or eight times, 1 ml each) by centrifugation ($10,000 \times g$, 5 min). Treated, washed cells were then FITC labeled and used in adhesion assays as described above.

Effect of pH on attachment. FITC-labeled cells (0.5×10^7 to 1.0×10^7 cells/ml) were washed twice and resuspended in one of the following physiological buffers (0.1 M) before they were used in adhesion assays: acetate (pH 4 and 5), morpholineethanesulfonic acid (MES) (pH 6 and 7), HEPES (pH 7 and 8), and TB (pH 8). Control wells with cells suspended in TB (pH 8) were used to determine initial cell concentrations due to the pH sensitivity of FITC fluorescence intensity measurement. Normalization due to pH effects on the FITC intensity was performed by allowing adhered cells (i.e., cells after the adhesion assay incubation and washing steps) to equilibrate in TB (pH 8) until the fluorescence intensity of the signal of the cells stopped increasing.

Competition assays. Cells were FITC labeled, and the final cell pellets resulting from the washing steps of the labeling reaction were suspended in TB containing either 0.3 M carbohydrate (added as a competitor), 0.1% detergent (SDS, Tween 80, or cetyltrimethylammonium bromide [CTAB]) or 1 M NaCl. Cells were then immediately used in adhesion assays.

Contact angle determination. Contact angle (c∠a) measurements for the surfaces used to evaluate the adhesive properties of the fungal cell types were obtained by using a Ramehart model 500 Advanced goniometer with an automated drop dispenser and tilting plate, using the DropImage Advanced software. Dynamic angle measurements were obtained just prior to movement of the water drop. Briefly, a 10-μl drop of sterile water was placed onto the surface of the substratum to be tested. The stage and the camera were tilted at 10° increments until the drop was on the verge of movement. The advanced (dynamic) contact angle was then determined.

Rodlet layer extraction. The rodlet layer proteins were removed from the surface of the spores as described by Paris et al. (29). Briefly, aerial conidia, blastospores, and submerged conidia were prepared as described above, resuspended in water, and sonicated at 140 W (3-mm-diameter microtip, 50% duty cycle) twice for 10 min using a Sonifier cell disrupter B-30 (Branson Ultrasons, Rungis, France). Unlysed cells and cell debris were removed by low-speed centrifugation ($10,000 \times g$, 10 min), and the supernatant was centrifuged for 1 h at $50,000 \times g$. The resultant pellet was boiled in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (2% SDS, 5% β-mercaptoethanol, and 10% glycerol in 62 mM Tris-HCl, pH 6.8) and washed twice with sample buffer and three times with distilled water. The final pellet was lyophilized and then treated with 100% trifluoroacetic acid for 10 min at room temperature. The acid was removed under a stream of nitrogen, and dried extracts were stored at room temperature under dry air and resuspended in water prior to analysis. Aliquots of a protein sample were mixed with 4× lithium dodecyl sulfate sample buffer (Invitrogen, Carlsbad, CA) plus dithiothreitol and run on a 10% bis-Tris NuPAGE gel with MES-SDS gel running buffer together with standards. Protein bands were visualized using either Sypro Ruby red (Bio-Rad, Hercules, CA) or Coomassie blue.

RESULTS

A quantitative assay was developed in order to measure the kinetics of fungal cell adhesion to various substrata. Fungal cells chemically treated with the fluorescent reagent FITC appeared to be uniformly labeled, with clear halo rings defining the cell envelope. Labeling of all three cell types, aerial conidia, blastospores, and submerged conidia, revealed a linear relationship between cell number (as measured by cell counting using a hemocytometer) and fluorescence intensity. Little variation was observed within experiments; however, some variation (up to a twofold difference in fluorescence intensity) was observed between experiments (i.e., between separate FITC labeling reactions). Therefore, a standard curve of fluorescence intensity versus cell number as determined by cell counting was determined and used for each experiment.

In order to determine the effects of the labeling reaction on the adhesive qualities of the cells, a series of preliminary qualitative experiments were performed using untreated and siliconized glass slides with both unlabeled and labeled cells. In all instances no difference was observed between using FITC-labeled cells and using unlabeled cells. These data demonstrated (i) that aerial conidia were able to bind to hydrophobic surfaces but not to hydrophilic surfaces; (ii) that blastospores bound uniformly to hydrophilic surfaces but bound poorly to hydrophobic surfaces, forming small clumps on the latter; and (iii) that submerged conidia bound equally well to both hydrophilic and hydrophobic surfaces, forming large clumps that appeared to become more evenly distributed over time. These patterns were identical for FITC-labeled and unlabeled cells.

For the quantitative assays, the following three types of black polystyrene-based microtiter plates with different surface characteristics were used as substrata: (i) siliconized Fluorotrac F200 plates, which were highly hydrophobic; (ii) F200 untreated polystyrene surface plates, which were weakly polar; and (iii) F600 plates, which were treated polystyrene and had hydrophilic, polar surfaces containing hydroxyl, carbonyl, and amino groups with a small net negative charge. Dynamic advanced water droplet contact angle measurements for the three substrata were consistent with the decreasing hydrophobicities; the silinated F200 plates displayed a c∠a of 104.7°, the untreated F200 plates displayed a c∠a of 95.6°, and the F600 plates displayed a c∠a of 85.6° (the contact angles for cleaned polished glass, the glass chamber slides, and silinated glass were determined to be 73.1°, 87.4°, and 109.7°, respectively).

The number of binding sites per microtiter plate well was estimated to be 4×10^5 to 8×10^5, as determined by the saturation point derived from plots of the percentage of cells bound as a function of the cell concentration (Fig. 1). These data indicated that the linear ranges of the cell types were

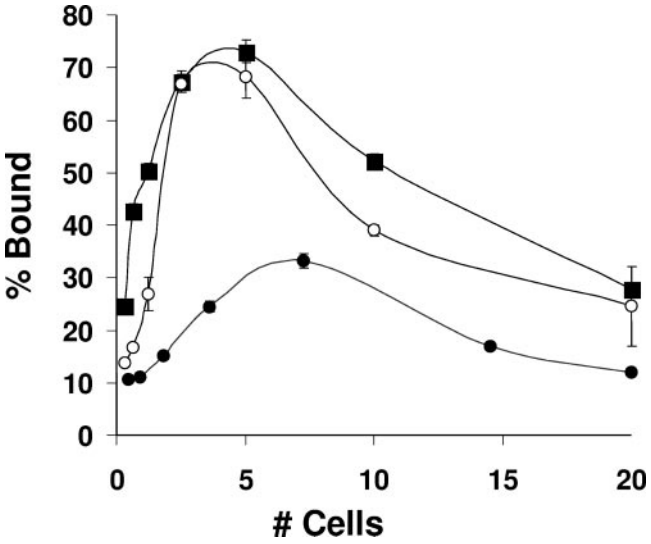

FIG. 1. Saturation points of sites for binding of *B. bassiana* aerial conidia (●), blastospores (■), and submerged conidia (○) to microtiter plates. The error bars indicate the results of at least three independent experiments.

similar, although the saturation points for the cell types varied from approximately 25% of the aerial conidial cells able to bind per well to more than 70% of the submerged conidia bound in wells when ~5×10^5 cells/well was used. All subsequent experiments were performed using cell concentrations in the linear range of the attachment curve (2×10^5 to 5×10^5 cells/well).

Quantitative adhesion assays using aerial conidia, blastospores, and submerged conidia on hydrophobic, weakly polar, and hydrophilic surfaces with either one or three washes were performed (Fig. 2). Aerial conidia bound rapidly and tightly to hydrophobic surfaces, and there was no decrease in cell binding after up to 10 washes with buffer (data not shown). Aerial conidia bound poorly to weakly polar surfaces even after prolonged exposure (24 h) to the substrata. Interestingly, these cells bound weakly to hydrophilic surfaces and were readily washed off. In contrast, blastospores bound poorly to hydrophobic surfaces, and no more than 10% of the cells were bound even after 24 h. Blastospores bound moderately to weakly polar surfaces, and approximately 1×10^5 to 2×10^5 cells bound/well (30% of the 5×10^5 cells used in the assay) after 4 h of incubation. Blastospores bound more readily to hydrophilic surfaces, and up to 50% of the cells bound within 30 min. Submerged conidia displayed the broadest binding characteristics, adhering to all three surfaces, although with slightly different kinetics. On hydrophobic, weakly polar, and hydrophilic surfaces, up to 60% of the cells used bound to the substrata within 4 h, although in the case of the hydrophobic and weakly polar surfaces, almost one-half of the bound cells could be removed using three washes.

Attachment of aerial conidia to hydrophobic surfaces could not be competed with any of the carbohydrate compounds tested, including glucose, galactose, lactose, maltose, melibiose, or trehalose, and was not sensitive to salt concentrations as high as 1 M NaCl (Table 1). The presence of *N*-acetylglucosamine appeared to promote adhesion (confirmed by microscopic analysis). The effect was not due to any visible growth or mucilage production; i.e., no germination, germ tube, or secretion of an extracellular matrix could be seen. Conidial attachment was, however, sensitive to the presence of detergents, and 80 to 90% inhibition was observed when 0.1% Tween 80 (a nonionic detergent), CTAB (a cationic detergent), or Triton X-100 (a nonionic detergent) was used; SDS (an anionic detergent) also inhibited conidial attachment to hydrophobic surfaces, although a large degree of variation was observed. Adhesion of aerial conidia to surfaces was only slightly affected by pH, and 30% fewer cells were bound at pH 4.0 than at pH 7.0.

A unique feature of blastospore attachment was that adhesion could be competed with maltose (Table 1). No other sugar tested had any effect on blastospore adhesion, nor did maltose affect conidial or submerged conidial adhesion. Blastospore attachment was not sensitive to salt (1 M NaCl), SDS, and CTAB, but it was inhibited by Tween 20 and Triton X-100. In contrast to the adhesion of the other cell types, the adhesion of blastospores appeared to be pH dependent, and there was a 50% decrease in the number of cells bound when assays were performed at pH 4 to 5 compared with the number of cells bound when assays were performed at pH 7 to 8. Submerged conidia behaved like aerial conidia, except that *N*-acetylglucosamine did not increase the number of cells bound and the presence of the detergents CTAB and Triton X-100 (Table 1) and changes in pH had only minor effects on microcycle conidial adhesion.

Removal of carbohydrates (maltose, glucose, or glucuronic acid) from the cell surface of aerial conidia using either α-amylase or laminarinase, but not removal of carbohydrates using cellulase, resulted in decreased conidial adhesion to hydrophobic surfaces but had no effect on conidial adhesion to hydrophilic surfaces (Table 2). Treatment of blastospores with glycosidases appeared to either slightly promote adhesion (α-amylase and to a lesser extent cellulase treatment) to hydrophilic surfaces or to not affect adhesion (laminarinase and/or hydrophobic surface). Glycosidase treatment of submerged conidia resulted in a 25 to 50% decrease in adhesion of cells to hydrophilic surfaces and hydrophobic surfaces except for α-amylase-treated cells, for which great variation was observed for adhesion to hydrophobic surfaces. Some differential effects were observed after protease treatment of the cell types. Aerial conidia treated with pronase E exhibited a more than 50% loss of adhesion to hydrophobic surfaces but no loss of adhesion to hydrophilic surfaces, although great variation was observed. This variation was between experiments (i.e., different cell batches treated with the enzyme) and may have reflected surface heterogeneity or accessibility of target substrates to the enzyme. Protease K treatment of aerial conidia did not result in appreciable changes in adhesion. Similar treatment of blastospores with proteases had no effect or resulted in an almost twofold apparent increase in adhesion. Microscopic analysis (i.e., visual counting) of the number of cells bound indicated that there did not appear to be an actual increase in the number of cells bound; instead, protease treatment appeared to increase the intensity of the fluorescence signal. These conditions were the only conditions tested in which the fluorescent signal was affected, and in all other experiments the results of microscopic analysis were in agreement with the fluorescent intensity measurements. Finally, little or no effect

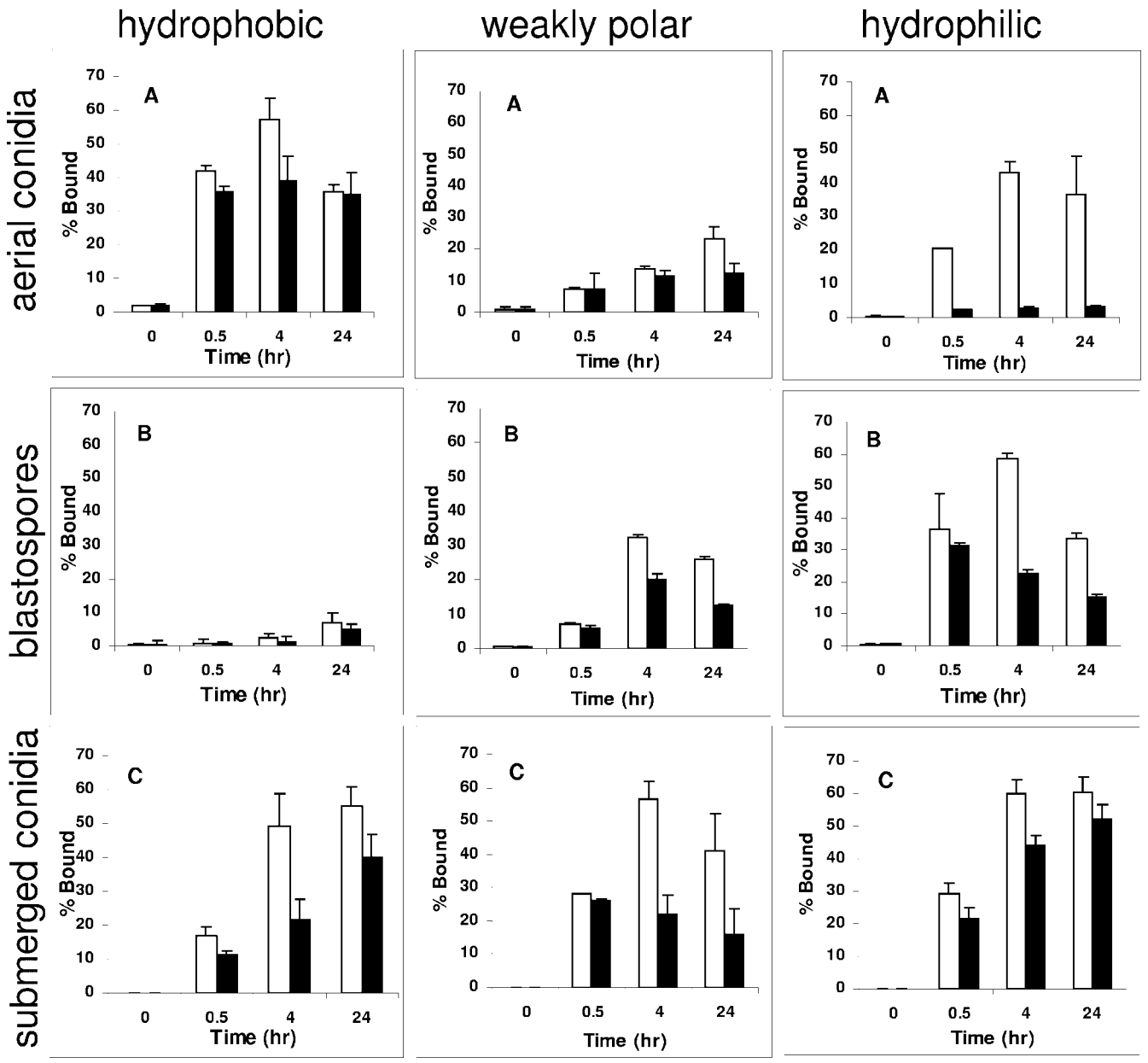

FIG. 2. Quantitative adhesion and influence of washing on adhesion of aerial conidia (A), blastospores (B), and submerged conidia (C) to silinated F200 (hydrophobic), F200 (weakly polar), and F600 (hydrophilic) microtiter plates. The percentages of cells bound after one wash (open bars) and after three washes (solid bars) are presented versus assay incubation time. The error bars indicate the results of at least three independent experiments.

was observed on the adhesion properties of protease-treated submerged conidia.

Hydrophobic interactions are thought to be mediated (at least in part) by low-molecular-weight proteins termed hydrophobins that form a rodlet layer on the surface of fungal cells. Hydrophobins are highly resistant to extraction by detergents but can be solubilized by (100%) trifluoroacetic acid. *B. bassiana* aerial conidia, blastospores, and submerged conidia were examined for the presence of hydrophobins by using cell extracts prepared as described in Materials and Methods and were analyzed by SDS-PAGE (Fig. 3) (gels stained with Sypro Ruby red gave essentially the same results). Protein bands corresponding to putative hydrophobins were extracted only from aerial conidia, and no bands were formed by extracts derived from either blastospores or submerged conidia.

DISCUSSION

Conidial adhesion has been examined in a number of plant- and insect-pathogenic fungi (2, 28). Adhesion of entomopathogenic fungi has been considered to involve an initial binding interaction followed by a consolidation step, resulting in firm attachment to the cuticle (2, 13, 14). Similarly, studies on the phytopathogenic fungus *Botrytis cinerea* revealed a two-stage

TABLE 1. Effects of various competitors and chemicals on *B. bassiana* cell adhesion

Compound	Attachment ratio[a]		
	Aerial conidia[b]	Blastospores[c]	Submerged conidia[c]
Glucose	1.0 ± 0.1	1 ± 0.1	1 ± 0.1
N-Acetylglucosamine	2.0 ± 0.1	1 ± 0.1	1 ± 0.1
Fucose	1.0 ± 0.2	1 ± 0.2	1 ± 0.1
Melibiose	1.0 ± 0.2	1 ± 0.2	ND[d]
Maltose	1.0 ± 0.2	0.1 ± 0.1	1 ± 0.3
Trehalose	0.8 ± 0.1	0.9 ± 0.1	1 ± 0.1
1 M NaCl	1.0 ± 0.2	0.9 ± 0.2	1 ± 0.2
0.2% Tween 20	0.2 ± 0.1	0.1 ± 0.1	ND
0.2% SDS	0.4 ± 0.5	0.8 ± 0.3	0.3 ± 0.1
0.2% CTAB	0.2 ± 0.1	0.6 ± 0.3	1 ± 0.2
0.2% Triton X-100	0.1 ± 0.1	0.3 ± 0.1	0.6 ± 0.1

[a] Attachment ratio = (% cells bound under conditions tested)/(% cells bound under control conditions). The data are the means ± standard deviations for at least three independent experiments.
[b] Cells were tested on siliconized F200 (hydrophobic) microtiter plates.
[c] Cells were tested on F600 (hydrophilic) microtiter plates.
[d] ND, not determined.

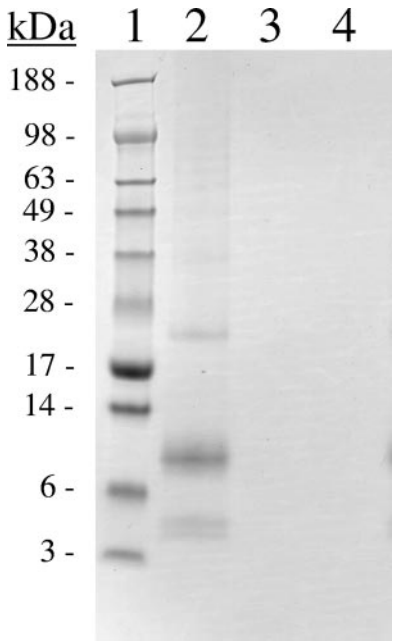

FIG. 3. Analysis of SDS-insoluble trifluoroacetic acid-soluble material from ultrasonic extracts of *B. bassiana* aerial conidia, blastospores, and microcycle conidia. An SDS-PAGE gel (10% polyacrylamide, bis-Tris) was stained with Coomassie blue. Lane 1, protein molecular weight standards; lane 2, aerial conidia; lane 3, blastospores; lane 4, submerged conidia.

adhesion process; immediate adhesion occurred upon hydration and was characterized by relatively weak attachment, and stronger delayed adhesion was observed as the conidia germinated (10, 11). Immediate adhesion of *B. cinerea* was passive (nonmetabolic), and although no specific structures were visible on the conidia, adhesion was characterized as dependent (in part) on hydrophobic interactions. Hydrophobic interactions have also been implicated in the attachment of conidia of the insect-pathogenic fungi *Nomuraea rileyi*, *Metarhizium anisopliae*, and *B. bassiana* to both host and nonhost cuticle preparations (3).

B. bassiana produces at least three single-cell types that can be distinguished based on morphological and adhesive characteristics. Qualitative studies assessing entomopathogenic fungal adhesion to various surfaces, including insect cuticles (2, 3, 10, 11, 17), have almost exclusively addressed conidial binding to surfaces and have not examined the adhesion properties of either blastospores or submerged conidia. We used a quantitative adhesion assay, and our results demonstrate that there are complex interactions between various cell types and substrata with different surface properties. All three *B. bassiana* single-cell types studied, aerial conidia, blastospores, and submerged conidia, displayed different adhesion properties that appeared to be mediated by different cell-specific mechanisms.

B. bassiana aerial conidia were able to bind to both hydrophobic and hydrophilic surfaces, although adhesion to the latter was weak and the cells could readily be washed off. Aerial conidia binding to hydrophobic surfaces could not be competed off with any carbohydrate tested, although addition of *N*-acetylglucosamine, the monomeric constituent of chitin, the major carbohydrate (polymer) found in arthropod cuticles, appeared to increase adhesion. The hydrophobic nature of *B. bassiana* conidial spores, as well as conidial spores from other entomopathogens, such as *N. rileyi*, *M. anisopliae*, and *Paecilomyces fumsoroseus*, has been correlated with the presence of an outer cell layer comprised of rodlets or fascicles, as visualized

TABLE 2. Effects of various enzymatic treatments on *B. bassiana* cell adhesion

Enzyme	Attachment ratio[a]					
	Aerial conidia		Blastospores		Submerged conidia	
	Hydrophobic[b]	Hydrophilic[c]	Hydrophobic	Hydrophilic	Hydrophobic	Hydrophilic
α-Amylase	0.3 ± 0.05	1.0 ± 0.3	1.2 ± 0.2	1.5 ± 0.15	1.4 ± 0.2	0.5 ± 0.15
Cellulase	0.8 ± 0.15	1.1 ± 0.2	1.0 ± 0.7	1.3 ± 0.05	0.4 ± 0.2	0.6 ± 0.15
Laminarinase	0.3 ± 0.05	0.9 ± 0.3	0.9 ± 0.1	1.0 ± 0.2	0.4 ± 0.2	0.8 ± 0.15
Pronase E	0.4 ± 0.3	1.2 ± 0.3	0.9 ± 0.1	1.7 ± 0.2[d]	1.1 ± 0.2	1.1 ± .05
Protease K	0.9 ± 0.2	0.8 ± 0.1	1.0 ± 0.1	1.8 ± 0.2[d]	2.8 ± 0.4	1.0 ± 0.15

[a] Attachment ratio = (% cells bound under conditions tested)/(% cells bound under control conditions). The data are the means ± standard deviations for at least three independent experiments, each consisting of triplicate determinations.
[b] Cells were tested on siliconized F200 (hydrophobic) microtiter plates.
[c] Cells were tested on F600 (hydrophilic) microtiter plates.
[d] Apparent increase (see text for details).

by electron microscopy (2, 3). The rodlet layers are presumably formed by assembly of specific proteins termed hydrophobins, which in turn are thought to passively mediate adhesion to hydrophobic surfaces (34, 35). Consistent with this model, extraction of the *B. bassiana* cell types revealed the presence of putative hydrophobins (a rodlet layer) in aerial conidia that were absent from both blastospores and submerged conidia. Although hydrophobins may account for some of the observed adhesion qualities, our data also indicate that the interaction of aerial conidia with hydrophobic surfaces may be more complex. Since amylase and laminarinase treatments, as well as protease treatments, reduced adhesion (but had no discernible effects on the rodlet layer [B. H. Kirkland, D. J. Holder, and N. O. Keyhani, unpublished results]), both carbohydrates on the cell surface and proteins may be involved in mediating adhesion of aerial conidia. Some caution, however, should be taken in any interpretation of enzymatic treatments of cells since amylase, laminarase, and cellulase are enzyme mixtures which contain various additional enzymes, often including proteases.

In contrast, blastospores, which are cells that lack any visible rodlet layer (2, 3) and from which no (putative) hydrophobins were extracted under the conditions tested, bound poorly to hydrophobic surfaces, forming small aggregates or clumps, but they displayed high levels of binding to hydrophilic substrata. Blastospores were also able to bind to weakly polar substrata, although the incubation time required was greater than that required for hydrophilic substrata. Intriguingly, blastospore attachment could be specifically competed with maltose. No other carbohydrate tested appeared to compete with adhesion of blastospores or the other fungal cell types; this included trehalose, the major carbohydrate constituent found in insect hemolymph. In vivo-generated blastospores (distinct but similar to the rich broth-produced blastospores), produced during fungal proliferation in the insect hemolymph after penetration of the cuticle, are able to evade recognition by insect hemocytes and display altered membrane characteristics (19, 20, 30); however, the physiological significance of potential maltose inhibition of adhesion of these cells is unclear.

Submerged conidia displayed the broadest binding characteristics of the *B. bassiana* single-cell types (they also appeared not to contain a rodlet layer or hydrophobins), and they were able to bind to hydrophobic, weakly polar, and hydrophilic surfaces. Spore tips or mucilage-covered appendages and adhesive knobs have been implicated as structures that mediate conidial adhesion of several fungi (1, 18, 33). The mucosal coat of nematophagous fungi not only appears to mediate adhesion but also is attractive to host insects, and a wide variety of arthropod mycopathogens appear to produce exocellular mucilage during germ tube or appressorial formation (2). Similarly, the hydrophilic nature of conidia of the Entomophthorales is thought to be mediated by a mucilaginous coat released upon attachment to cuticle surfaces, which acts as a glue mediating attachment (12, 24). Although blastospores and submerged conidia attached to hydrophilic surfaces, no obvious mucilaginous coat was visible in either cell type, and scanning electron microscopy did not reveal any specific structures in either conidia, blastospores, or submerged conidia of *B. bassiana* that appeared to be involved in mediating adhesion (unpublished data). It is possible, however, that extracellular matrix components or mucilage located between the inner and outer walls that may not be readily detectable could be involved in mediating adhesion.

Although aerial conidia are considered easily dispersible via air currents and due to their spore-like cell walls are more resistant to adverse environmental conditions, such as desiccation and extreme temperatures, microcycle conidiation and blastospore formation may occur under a variety of environmental conditions, as well as during the host-pathogen interaction. The production of multiple cell types with different adhesive properties may occur in response to specific environmental conditions, allowing fungal cells to bind to a broad range of host targets and providing the fungus a way to adapt to substratum conditions (13, 14). It is unlikely, however, that alteration of adhesion can account for the emergence of the restricted-host-range *B. bassiana* strains since these strains may have altered (cuticle-degrading) enzyme production or be unable to penetrate and/or respond to surface cues of certain hosts but still retain the means to initiate binding or adhesion interactions. Indeed, there is some evidence that when entomopathogens specialize, they lose structures rather than gain them, although it would be interesting to see whether any alteration in the adhesion kinetics of general and specialized strains of *B. bassiana* occurs. Our data do indicate that certain practical considerations should be taken into account during application of *B. bassiana*. For instance, if blastospores are to be used, formulations should probably contain aqueous or nonpolar liquids that may prove to be more successful in biocontrol of certain hosts compared to aerial conidia. In contrast, the use of detergents in order to avoid aggregation of aerial conidia may prove to be detrimental during application since the presence of such detergents can decrease adhesion to surfaces. Future research correlating the virulence of the fungal cell types to specific insect targets may lead to rational design decisions for the selection of fungal strains with greater specificity for desired targets.

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Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii*

Gisbert Zimmermann[a]
[a] Federal Biological Research Centre for Agriculture and Forestry, Institute for Biological Control, Darmstadt, Germany

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REVIEW

Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii*

GISBERT ZIMMERMANN

Federal Biological Research Centre for Agriculture and Forestry, Institute for Biological Control, Darmstadt, Germany

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Abstract
The commercial use of entomopathogenic fungi and their products as mycoinsecticides necessitates their registration. Worldwide, several registration guidelines are available, however, most of them focus on similar or even the same safety issues. With respect to the two entomopathogenic fungi, *Beauveria bassiana* (Bals.-Criv.) Vuill. and *Beauveria brongniartii* (Sacc.) Petch, many commercial products have been developed, and numerous papers on different biological, environmental, toxicological and other safety aspects have been published during the past 30–40 years. The aim of the present review is to summarise these data. The following safety issues are presented: (1) identity of *Beauveria* spp.; (2) biological properties of *Beauveria* spp. (history, natural occurrence and geographical distribution, host range, mode of action, production of metabolites/toxins, effect of environmental factors); (3) analytical methods to determine and quantify residues; (4) fate and behaviour in the environment (mobility and persistence in air, water and soil); (5) effects on non-target organisms (non-target microorganisms, plants, soil organisms, aquatic organisms, predators, parasitoids, honey bees, earth worms and nontarget arthropods); (6) effects on vertebrates (fish, amphibia, reptiles and birds); and (7) effects on mammals and human health. Based on the present knowledge it is concluded that both *Beauveria* species are considered to be safe.

Keywords: Beauveria bassiana, Beauveria brongniartii, *occurrence, host range, toxins, environmental fate, safety, side-effects*

Introduction

The two entomopathogenic fungal species *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin and *Beauveria brongniartii* (Saccardo) Petch were described for the first time about 170 and 110 years ago, respectively. Since that time they have always been

Correspondence: Gisbert Zimmermann, Federal Biological Research Centre for Agriculture and Forestry, Institute for Biological Control, Heinrichstrasse 243, D-64287 Darmstadt, Germany. Tel: +49 6151 4070. Fax: +49 6151 407 290. E-mail: gisbert.zimmermann@gmx.net

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considered as fungi that can and should be used for control of pest insects. In the early days of biological control and especially microbial control, there was no concern for possible side-effects or safety considerations of these two species. Steinhaus (1957) was possibly the first who raised questions on the safety of microbial control products to man, other vertebrates and even to crops. He very carefully discussed different aspects of the scientific knowledge at that time. Although he concluded that microorganisms pathogenic to insects are in general harmless to man, animals and plants, he recommended that such products are subjected to appropriate State and Federal regulations. A few years later, Müller-Kögler (1965) published a book (unfortunately in German) on fungal diseases of insects, practical use for biological control and basics of insect mycology, in which some sections on side-effects of entomopathogenic fungi on humans and other warm-blooded animals as well as on beneficial insects were already included. In 1971, Heimpel summarised the knowledge on safety of insect pathogens, i.e. bacteria, viruses, protozoa, fungi and rickettsiae for man and vertebrates. He also emphasised the necessity for testing the safety of insect pathogens and said 'it seems incredible that so many good scientists have worked so long with insect pathogens without testing them for safety . . .'. He also mentioned registration guidelines of the USA and other countries at that time. A similar review was published two years later by Ignoffo (1973).

With the increasing interest in biological control of pest insects between 1980 and 1990, safety aspects were discussed in more detail (e.g. Austwick 1980; Burges 1981; Hall et al. 1982; Laird et al. 1990). Burges (1981) outlined the main principles and guidelines for testing the safety of insect pathogens and stated 'I believe that a pathogen should be registered as safe when there is reasonable evidence that it is so and in the absence of concrete evidence that it is not. A "no risk" situation does not exist, certainly not with chemical pesticides, and even with biological agents one cannot absolutely prove a negative'. First guidelines for the registration of entomopathogenic fungi under the auspices of the IOBC as an advisory document were published by Hall et al. (1982). The first book, dedicated alone to the safety of microbial insecticides was published by Laird et al. (1990), including sections on safety to domestic animals and wildlife (Saik et al. 1990) and to vertebrates and humans (Siegel & Shadduck 1990). In 1996, Cook and coauthors published an interesting framework for scientific safety evaluation of microorganisms intended for pest and plant disease control. The intention was to identify and discuss safety issues linked to microbial control agents which should stimulate and improve discussions on possible risks and risk management. Later reviews on safety of entomopathogenic fungi and especially also on *Beauveria* spp. were published by Goettel and Jaronski (1997), Goettel et al. (2001), Vestergaard et al. (2003) and Copping (2004). A good summary of various safety issues of *B. bassiana* strain GHA (128924) and strain ATCC 74040 (128818) can be found at www.epa.gov/pesticides/biopesticides/ingredients/factsheets/factsheet_128924.htm and 128818.htm.

Within the EU, safety regulations are documented in the Directive 91/414/EEC. A national registration of a microbial product is only possible after extensive testing (and inclusion of the microorganism in Annex I of the guideline). Annexes IIB and IIIB to Directive 91/414/EEC set out the requirements for the dossier to be submitted by an applicant, respectively, for the inclusion of an active substance consisting of micro-organisms or viruses in Annex I to that Directive and for the authorisation of a plant protection product based on preparations of micro-organisms or viruses. A guidance

for registration requirements of microbial pesticides is also published by the OECD (Anon. 2003).

The aim of the present review is to summarise and discuss our present knowledge on the safety, possible side-effects and the environmental behaviour of both *Beauveria* species, *B. bassiana* and *B. brongniartii*, as basis for further discussions within the registration process and the intended use of these fungi as mycoinsecticides.

Identity of *Beauveria* spp.

In 1954, MacLeod published the first, careful review of literature on the genera *Beauveria* and *Tritirachium*, including a taxonomic revision of both genera. The studies comprise cultural and morphological characteristics of numerous *Beauveria* isolates and species. Fourteen of formerly described species of *Beauveria* were described as synonyms of *B. bassiana* and *B. tenella. Beauveria stephanoderis*, *B. laxa*, *B. globulifera*, *B. effusa*, *B. vexans*, *B. doryphorae*, *B. delacroixii* and *B. acridiorum* were included as strains of *B. bassiana*, while *B. densa*, *B. melolonthae*, *B. brongniartii* and *B. shiotae* are strains of *B. tenella*. Later, De Hoog (1972) restricted the genus *Beauveria* Vuill. to three species: *B. bassiana*, *B. brongniartii* and *B. alba*. A comprehensive description of the species is presented, including a key to species, morphological characteristics, a huge list of former synonyms and some representative figures. The main synonymous names of *B. bassiana* and *B. brongniartii* are presented in Table I.

Further descriptions of *Beauveria* spp. are presented by Domsch et al. (1980) and Humber (1997). Recently, the taxonomy and phylogenetics of the genus *Beauveria* was discussed in detail (Rehner 2005; Rehner & Buckley 2005). *Beauveria bassiana* is characterised by white, later yellowish or occasionally reddish colonies. The reverse is uncoloured, or yellowish to pinkish. Conidiogenous cells consist of globose to

Table I. Main synonyms of *B. bassiana* and *B. brongniartii* (MacLeod 1954; De Hoog 1972; CABI Bioscience et al. 2006).

Fungus	Synonym
B. bassiana (Balsamo-Crivelli) Vuillemin (1912)	*Beauveria laxa* Petch (1931)
	Beauveria globulifera (Speg.) Picard (1914)
	Botrytis bassiana Balsamo (1835)
	Botrytis necans Massee (1914)
	Botrytis bassiana Sacc. subsp. *tenella* Delacroix (1937)
	Botrytis bassiana var. *lunzinensis* Szilvinyi (1941)
	Botrytis brongniartii subsp. *delacroixii* (Sacc. Delacroix (1893)
	Botrytis effusa Beauverie (1911)
	Botrytis stephanoderis Bally (1923)
	Sporotrichum densum Link (1809)
	Sporotrichum globuliferum Speggazzini (1880)
	Sporotrichum minimum Speggazzini (1881)
	Isaria shiotae Kuru (1931)
B. brongniartii (Saccardo) Petch (1926)	*Beauveria densa* Link (1914)
	Beauveria tenella (Sacc.) McLeod sensu McLeod (1954)
	Botrytis tenella Saccardo (1874)
	Botrytis brongniartii Saccardo (1892)
	Botrytis melolonthae (Sacc.) Ciferri (1929)
	Isaria densa Link (1892)
	Sporotrichum epigaeum Daszew (1912)

flask-shaped basal part and an up to 20-µm long rachis, mostly forming a zig-zag. Conidia are hyaline, globose to broadly ellipsoidal, generally 2–3 × 2–2.5 µm. The conidia are formed in clusters, like snow balls or cotton balls. *Beauveria brongniartii* is characterised by first white, later yellowish to pinkish or reddish colonies. The reverse is uncoloured, yellowish or pinkish. The conidiogenous cells also consist of a subglobose or flask-shaped basal part with a long rachis. In contrast to *B. bassiana*, the hyaline conidia are ellipsoidal, (2 −) 2.5–4.5 (−6) µm. Conidia are clustered, but also arranged in small groups or are solitary.

Recent investigations have demonstrated that there is a direct link between the genus *Beauveria* and the teleomorph genus *Cordyceps* (Ascomycota: Hypocreales, Clavicipitaceae) (Shimazu et al. 1988; Bo et al. 2002; Liu et al. 2002; Rehner 2005; Rehner & Buckley 2005). However, all *Beauveria* teleomorphs have been described so far from Asia.

Biological properties of *Beauveria* spp.

History

The most comprehensive study on the genera *Beauveria* and *Tritirachium*, the history of the genus *Beauveria* and of its species and the cultural and morphological characteristics are presented in detail by Steinhaus (1949), MacLeod (1954) and, later, by De Hoog (1972). The early history of *B. bassiana* started in 1835. It was Agostino Bassi di Lodi from Italy, who was the first to show that a fungus can cause a disease in insects, thus enunciating the germ theory of disease. He observed a disease in silkworms, *Bombyx mori*, which he called 'white muscardine' and started the first infection experiments. The fungus was then studied and described by the famous Italian naturalist Giuseppe Gabriel Balsamo-Crivelli in 1835, who gave it the name *Botrytis bassiana*, in honour of Bassi (Steinhaus 1949; Müller-Kögler 1965; Rehner 2005).

In 1911, Beauverie studied the fungus again and in 1912 Vuillemin created the new genus *Beauveria* in honor of Beauverie, of which the species *B. bassiana* became the type. *Beauveria brongniartii* has been described under different names by several investigators at the end of the 19th century (MacLeod 1954). An extensive study of this fungus with respect to its use against *Melolontha melolontha* was already published more than 100 years ago by Giard (1892).

Since the first descriptions of the genus *Beauveria*, both *B. bassiana* and *B. brongniartii* were used in biocontrol against pest insects. In a section on 'Fungous Infections', Steinhaus (1949) summarised the infection process, the development of the disease and the practical use of *B. bassiana* against some pest insects, mainly the European corn borer, *Ostrinia* (*Pyrausta*) *nubilalis*, and the codling moth, *Carpocapsa pomonella*. He also mentioned practical use of *Beauveria globulifera*, which later was included in *B. bassiana*, against the chinch bug, *Blissus leucopterus*.

In the book of Müller-Kögler (1965), 28 species and families of pest insects in agriculture, orchards, forestry, greenhouse and in the tropics were mentioned against which *B. bassiana* had been used for control purposes. The fungus *B. brongniartii* (=*B. tenella*) however, was applied mainly against *Melolontha* spp. and *Epilachna vigintioctomaculata*. A review on pest control of the fungi *Beauveria* and *Metarhizium* including basic as well as practical aspects was published later by Ferron (1981). At that time, the *B. bassiana* product 'Boverin' was extensively used in the USSR on

thousands of hectars mainly against the Colorado potato beetle, *Leptinotarsa decemlineata*, and the codling moth *Cydia pomonella*, while *B. brongniartii* was applied against *M. melolontha*. In China, *B. bassiana* was produced and widely used against *O. nubilalis* in corn, *Dendrolimus punctatus* on pines and *Nephotettix* leafhoppers on rice and tea (Hussey & Tinsley 1981).

The present state of *Beauveria* products registered or under commercial development is summarised in Table II (Butt et al. 2001; Wraight et al. 2001; Copping 2004; Zimmermann 2005; Kabaluk & Gazdik 2005).

Natural occurrence and geographical distribution

Beauveria bassiana is the most widely distributed species of the genus. It is generally found on infected insects both in temperate and tropical areas throughout the world. MacLeod (1954) mentioned that *B. bassiana* was isolated from 63 different insect species collected in various localities throughout Canada. He also reported that *Beauveria* strains were found within the lung tissues of 14 rodents. However, histological examination did not show that the fungus creates a pathological condition within the tissues.

The occurrence and distribution of *B. bassiana* and *B. brongniartii* in various countries and areas are listed by Domsch et al. (1980). Reports of the occurrence of *B. bassiana* are from Turkey, the Ivory Coast, equatorial West Africa, central Africa, South Africa, the Bahamas, Nepal, east Siberia, New Zealand and Japan. Habitats for *B. bassiana* range from an alpine soil, to heathland, peat bogs, soils with savannah type vegetation, forest and cultivated soils, sand blows and dunes, desert soil and running

Table II. Mycopesticides of *Beauveria bassiana* and *B. brongniartii* registered or under commercial development (Butt et al. 2001; Wraight et al. 2001; Copping 2004; Kabaluk & Gazdik 2005; Zimmermann 2005).

Fungus	Product/Trade name	Company/Producer	Country/Origin
B. bassiana	Bio-Power	Stanes	India
	BotaniGard ES	Laverlam International (formerly	USA
	BotaniGard 22WP	Emerald BioAgriculture)	
	Boverol	Fytovita	Czech Republic
	Conidia	LST	Columbia
	Mycotrol ES	Laverlam International	USA
	Mycotrol-O	(formerly Emerald BioAgriculture)	
	Naturalis	Intrachem	Italy
	Naturalis-L	Andermatt Biocontrol	Switzerland
		Troy Biosciences Inc.	USA
	Ostrinil	Arysta (formerly NPP, Calliope)	France
	Proecol	Probioagro	Venezuela
	Racer BB	SOM Phytopharma	India
	Trichobass-L	AMC Chemical/Trichodex	Spain
	Trichobass-P		
B. brongniartii (=*B. tenella*)	Beauveria Schweizer	Lbu (formerly Eric Schweizer Seeds)	Switzerland
	Betel	Arysta (formerly NPP, Calliope)	France
	Biolisa-Kamikiri	Nitto Denko	Japan
	Engerlingspilz	Andermatt Biocontrol AG	Switzerland
	Melocont-Pilzgerste	Agrifutur-Kwizda	Italy–Austria

water. Also, isolations from the rhizoplane of peat bog plants, the rhizosphere of clover, dead bark, nests, feathers and droppings of free-living birds were mentioned.

Beauveria brongniartii is less common than *B. bassiana*, but also has a worldwide distribution in insects as well as in different habitats. It has been reported from open bogs, alpine habitats, forest soil in Hong Kong, terra rossa in Greece, a *Calluna* heath, an alpine grassland in Italy and sand dunes in the British Isles (see Domsch et al. 1980).

Beauveria bassiana has also been isolated from the surface and the interior of plants. Using selective media, *B. bassiana* was isolated from bark of elm trees and from soil at the base of elm trees (Doberski & Tribe 1980) and from the bark of *Carpinus caroliniana* (ironwood, hop hornbeam) (Bills & Polishook 1991). Recently, the species was also found naturally occurring on phylloplanes of various hedgerow plants (Meyling & Eilenberg 2006). Further information on the natural occurrence of *B. bassiana* in general and as an endophyte in various plant species are reported in *Host range* and *Effects on Plants*, respectively.

The description of the so-called 'Galleria-bait-bait' (Zimmermann 1986) and various selective media for isolation of *Beauveria* spp. from soil (see *Analytical methods to determine and quantify residues*) have lead to a huge increase in the findings of *B. bassiana* throughout the world (Table III).

Although *Beauveria* spp. are no common airborne fungi, *B. bassiana* has been isolated from the air. In a study on fungal biodiversity in the air of Turin, Italy, the species was found at a mean of 0.2 CFU m^{-3} during 10 months per year, while *B. brongniartii* was found at only <0.1 CFU m^{-3} during one month (Airaudi & Filipello-Marchisio 1996). The natural density of *B. bassiana* in the air of a forest in Japan ranged from 0 to 3.1×10^3 CFU m^{-2} day^{-1} (Shimazu et al. 2002). In the air of a hospital, the fungus was isolated together with 98 other fungal species (Rainer et al. 2000). Nolard (2004) mentioned *B. bassiana* in the air of humid dwellings of allergic patients. For natural occurrence in vertebrates including mammals and humans (see *Effects on vertebrates* (*fish, amphibia, reptiles and birds*) and *Effects on mammals and human health*). Recently, both *Beauveria* spp. were found in surface water-derived drinking water in Norway, but not in groundwater derived water (Hageskal et al. 2006).

Host range

Beauveria bassiana is a ubiquitous entomopathogenic fungus which has been found and isolated from a wide variety of insects from different orders (MacLeod 1954; Leatherdale 1970; Li 1988; Goettel et al. 1990). MacLeod (1954) mentioned about 60 insect species from which *Beauveria* strains have been isolated. The hosts of 106 species of entomopathogenic fungi known from Britain are catalogued by Leatherdale (1970). Listed hosts of *B. bassiana* are Heteroptera (*Picromerus bidens*, *Anthocoris nemorum*), Homoptera (*Eulecanium* spp.), Lepidoptera (*Hepialus* spp., *Hypocrita jacobaea*, *Cydia nigricans*), Coleoptera (*Lathrobium brunnipes*, *Calvia quattuordecimguttata*, *Phytodectra olivacea*, *Otiorhynchus sulcatus*, *Sitona lineatus*, *S. sulcifrons*, *S. macularius*, *S. hispidulus*, *Anthonomus pomorum*, *Hylaster ater*), Hymenoptera (Ichneumonidae, *Lasius fuliginosus*, *Vespula* spp., *Bombus pratorum*), Diptera (*Leria serrata*) and spiders.

Table III. Natural occurrence of *Beauveria bassiana* and *Beauveria brongniartii* in the soil in different countries and areas.

Location	Results	Reference
Canada	In 266 soil samples from 86 locations the most abundant species were *B. bassiana* (187 isolates) and *M. anisopliae* (357 isolates)	Bidochka et al. (1998)
Czech Republic: South Bohemia; arable soil	From 146 soil samples 25 strains of *B. bassiana* were isolated; no differences in soils from arable fields on conventional and organic farms	Landa et al. (2002)
Finland	From 590 soil samples, *B. bassiana* was isolated from 19.8%	Vänninen (1996)
Germany	In 100 soil samples from different locations and soil types, *B. bassiana* was found in 22%	Kleespies et al. (1989)
Italy: Southern part	In 188 soil samples, the most common entomopathogen was *B. bassiana*	Tarasco et al. (1997)
Japan	*B. bassiana* was often isolated from forest soils	Shimazu et al. (2002)
Macquarie Islands	In 163 subantarctic soils samples, 1 contained *B. bassiana*	Roddam and Rath (1997)
Nepal	*B. bassiana* was isolated from a few soil samples	Dhoj and Keller (2003)
New Zealand	*B. bassiana* was higher in pasture soils than in forest or cropland soils	Barker and Barker (1998)
Norway: Northern parts	Significantly higher occurrence of entomopathogenic fungi in soils from arable fields of organically managed farms compared to conventionally ones. Species found were *B. bassiana*, *M. anisopliae* and *Tolypocladium cylindrosporum*	Klingen et al. (2002)
Panama: Tropical forest	*B. bassiana* was detected in soil near colonies of leaf-cutting ants	Hughes et al. (2004)
Poland: Apple and plum orchards	*B. bassiana* was dominant in soil under sward in both kinds of orchards	Sapieha-Waszkiewicz et al. (2003)
Poland: Hop plantations and arable fields	*B. bassiana* in all soil types and areas available	Mietkiewski et al. (1996)
Poland: Mid-forest meadows (Sudety mountains)	*B. bassiana* and *B. brongniartii* were isolated from various areas	Mietkiewski et al. (1994)
Poland: Different soil types	*B. bassiana* was the dominant species in muck and loess	Tkaczuk and Mietkiewski (1996)
Spain: Alicante	*B. bassiana* was most frequently found in 21% of soils from 61 sites	Asensio et al. (2003)
Switzerland	Soil samples from 82 fields were analysed: *B. brongniartii* was limited to soil sites colonised by its host, *Melolontha melolontha*; *B. bassiana* was also isolated	Keller et al. (2003)
USA	Soil from 105 sites in 21 orchards: From 16 orchards mainly *B. bassiana* and *M. anisopliae* were isolated	Shapiro-Ilan et al. (2003)

Based on worldwide data, Li (1988) listed 707 species of insect hosts of *B. bassiana*. These comprise 521 genera and 149 families of 15 orders. In addition, 13 host species of Acarina distributed in seven genera and six families are listed. The insect orders in which *B. bassiana* has been found as a pathogen are as follows:

(1) Lepidoptera, (2) Coleoptera, (3) Hymenoptera, (4) Homoptera, (5) Diptera, (6) Hemiptera, (7) Orthoptera, (8) Siphonaptera, (9) Isoptera, (10) Thysanoptera, (11) Mantodea, (12) Neuroptera, (13) Dermaptera, (14) Blattariae, (15) Embioptera.

The host range and specificity of *B. bassiana* is listed by Goettel et al. (1990) as follows: Gastropoda, Acari, Orthoptera, Dermaptera, Isoptera, Blattaria, Thysanoptera, Homoptera, Heteroptera, Diptera, Coleoptera, Hymenoptera, Siphonaptera and Lepidoptera. Furthermore, several hundred *B. bassiana* isolates from numerous host insects are listed in the USDA ARS Entomopathogenic Fungus collection.

However, despite the prevalence of *B. bassiana* in a huge number of arthropods, it is known that most isolates of this fungus have a restricted host range (Goettel et al. 1990; Vestergaard et al. 2003), and there are several examples that *B. bassiana* isolates from a distinct host insect or from the soil are highly virulent against other target pests (Feng & Johnson 1990; Ekesi et al. 1998; Cottrell & Shapiro-Ilan 2003). Therefore, it is necessary to screen the virulence of different isolates against a target insect species in order to select the most virulent one.

In Europe, *B. brongniartii* mainly attacks the field and the forest cockchafer, *Melolontha melolontha* and *M. hippocastani*. However, the fungus may also occur on other insects. According to Leatherdale (1970), the listed hosts of *B. brongniartii* (=*B. tenella*) are: Heteroptera (Pentatomidae), Lepidoptera (*Hepialus lupulinus*), Coleoptera (Coccinellidae, Chrysomelidae, *Plateumaris braccata*, *Galerucella tenella*, Curculionidae, *Strophosomus sus*.), Hymenoptera (Formicidae, *Vespula* spp.) and spiders. Vestergaard et al. (2003) summarised the reports on the occurrence of *B. brongniartii* and mentioned the following hosts: Coleoptera (Cerambycidae, Curculionidae, Ipidae, Lucanidae, Nitidulidae), Lepidoptera (Pyralidae), Homoptera (Cicadidae), Hymenoptera (Vespidae), Phasmatodea and Orthoptera. However, isolates from non-coleopteran hosts have a large genetic distance to those from *Melolontha* spp. (Enkerli et al. 2001).

Mode of action

There is a huge number of publications dealing with the mode of action and the infection process. The first publications dealing with the infection of certain pest insects by *B. bassiana*, such as the silkworm, were compiled by Steinhaus (1949) and Müller-Kögler (1965). Therefore, in this section only the general aspects of the infection process are summarised (see e.g. Boucias & Pendland 1998 for more specific information). A comprehensive overview on the biochemical aspects of disease development, its physico-chemical aspects and the genetics and molecular biology of disease development is presented by Khachatourians (1998).

As in other entomopathogenic fungi, *Beauveria* species attack their host insects generally percutaneously. The infection pathway consists of the following steps: (1) attachment of the spore to the cuticle; (2) germination; (3) penetration through the cuticle; (4) overcoming the host response and immune defense reactions; (5) proliferation within the host by formation of hyphal bodies/blastospores, i.e. yeast like cells; (6) saprophytic outgrowth from the dead host and production of new conidia.

Attachment is due to the hydrophobicity of the conidia as well as the cuticular surfaces. In *B. bassiana*, conidia contain a hydrophobin-type protein on their exterior surfaces. Germination and successful infection depends on a number of factors, e.g.

susceptible host and host stage and certain environmental factors, such as optimal temperature and humidity. Germination is further influenced by certain cuticular lipids, such as short-chain fatty acids, aldehydes, wax esters, ketones and alcohols which may possess antimicrobial activity. However, the cuticle may also be coated with substances that are important for fungal recognition, like free amino acids or peptides, and may trigger attachment and germination. Generally, germination of *B. bassiana* conidia starts after about 10 h and is largely completed by 20 h at 20–25°C.

Generally the fungus penetrates thinner, non-sclerotised areas of the cuticle, like joints, between segments or the mouthparts. Before penetration, germ tubes may produce so-called appressoria and infection pegs. The penetration process is by mechnical means and by the production of several enzymes, including proteases, chitinases and lipases.

The penetration of the cuticle layers and the beginning of invasion is accompanied by several host response activities, e.g. by production of phenoloxidase and certain hemocytes and melanisation. The interactions between the penetrating fungus and the insect immune system are a complex process and comprise many molecular and cellular reactions (Vilcinskas & Götz 1999). During the infection process, *Beauveria* spp. produce proteolytic enzymes and toxins, while the host insects respond with cellular and humoral defence reactions. These reactions consist of the production of antifungal compounds, inducible protease inhibitors and proteins, which detoxify fungal toxins in the insect.

After successful penetration, the fungus produces hyphal bodies, i.e. yeast like cells, which are distributed passively in the hemolymph, enabling the fungus to invade other tissues of the host insect by extensive vegetative growth and the production of toxins. For example, during this stage, the metabolite oosporein is produced by *B. brongniartii*, which is visible by turning its host cadaver red. During its invasion of the insect body, the fungus depletes nutrients in the hemolymph and the fat body. This process is followed by the death of the insect and the end of the pathogenesis (Boucias & Pendland 1998).

The incubation period depends on the host, the host stage, temperature and virulence of the fungus strain. In aphids, it may take 3–4 days, while in scarab larvae, 2–4 weeks. After the host death and under humid conditions, the fungus starts its saprophytic phase by emerging out of the host body and producing conidia on the exterior surface of the cadaver. Under very dry conditions, the fungus may also persist in the hyphal stage inside the cadaver or, e.g. in locusts in Africa, produce its conidia inside the body.

During the incubation period, the fungus may affect its host insect throughout behavioural and feeding changes, the reduction of body weight or fecundity, malformations or behavioural fever (Müller-Kögler 1965; Ekesi 2001; Ouedraogo et al. 2003).

Production of metabolites/toxins

Microorganisms, and especially fungi, produce a wide variety of compounds or metabolites, mostly within their secondary metabolism, which generally have diverse activities and functions. Therefore, it is not surprising that entomopathogenic fungi are also able to produce different metabolites. With respect to registration and risk assessment, these metabolites and their special activities are of particular toxicological

interest. In the following chapter, the most important metabolites of *B. bassiana* and *B. brongniartii* are presented and their occurrence and general activities are outlined.

One of the first comprehensive overviews on the toxins of entomopathogenic fungi is presented by Roberts (1981). The author summarises the knowledge of toxic metabolites produced by well-known entomopathogenic Deuteromycetes, such as *Beauveria*, *Metarhizium*, *Nomuraea, Aspergillus*, *Paecilomyces* and *Verticillium*, the ascomycete *Cordyceps* and the genus *Entomophthora* sensu lato. He presented four principle objectives of studies on fungal metabolites toxic to insects, which are still important: (1) to elucidate the mode of action; (2) to search for new chemicals for use in pest control; (3) to evaluate the safety of specific fungi proposed for use in pest control; and (4) to conduct basic chemistry studies on natural products. Today, we should add the search for new drugs and pharmaceuticals in human medicine. These objectives demonstrate that fungal metabolites generally cannot only be considered as safety issues. A recent review on toxic metabolites of entomopathogenic fungi including those used for other biocontrol purposes was given by Vey et al. (2001).

Both *Beauveria* spp. produce several toxic compounds *in vitro* and *in vivo* (e.g. Mazet et al. 1994; Strasser et al. 2000; Vey et al. 2001). These are presented in Table IV. A majority of these insecticidal molecules are low molecular weight secondary metabolites, mainly cyclic peptides such as beauvericin and bassianolide, and the pigments bassianin and tenellin. There is also evidence that melanising macromolecular toxins are secreted during mycosis in the haemolymph (Fuguet & Vey 2004). One toxic macromolecule was identified as a hydrophilic, chitosanase-like protein (Fuguet et al. 2004). Additionally, the secondary metabolite cyclosporin A is produced by *B. bassiana*, which was originally found in *Tolypocladium inflatum* (Boucias & Pendland 1998). The main metabolite produced by *B. brongniartii* is oosporein.

Beauvericin is the most important compound which was reported first from *B. bassiana.* Beauvericin is a toxic cyclic hexadepsipeptide and comprises a cyclic repeating sequence of three molecules of *N*-methyl phenylalanine alternating with three molecules of 2-hydroxyisovaleric acid. Not all isolates of *B. bassiana* produce beauvericin *in vitro* (for corresponding literature see Roberts, 1981). A review on the activity of beauvericin and two other metabolites, bassianolide and beauveriolide, is given by Strasser et al. (2000) and Vey et al. (2001). Beauvericin has also been isolated from other fungi, such as *Paecilomyces fumosoroseus* (see Roberts 1981), *Paecilomyces tenuipes* (Nilanonta et al. 2000) and, especially from members of the genus *Fusarium*

Table IV. Major metabolites produced by *Beauveria* spp.

Fungus	Metabolite	Reference
B. bassiana	Beauvericin, bassianin, bassianolide, beauverolides, beauveriolides, tenellin, oosporein	Strasser et al. (2000); Vey et al. (2001)
	Oxalic acid	Roberts (1981)
	Bassiacridin	Quesada-Moraga and Vey (2004)
B. brongniartii	Oosporein	Strasser et al. (2000); Vey et al. (2001)
	Oxalic acid	Müller-Kögler (1965); Roberts (1981)
B. felina	Destruxin B	Kim et al. (2002)

(e.g. Gupta et al. 1991; Bottalico et al. 1995; Logrieco et al. 1998). Gupta et al. (1991) detected beauvericin in cultures of *Fusarium moniliforme* var. *subglutinans* and *F. semitectum*, and later, a co-occurrence of beauvericin with fumonisin B_1 in *F. moniliforme* was reported (Bottalico et al. 1995). From 94 *Fusarium* isolates tested belonging to 25 taxa, the following species produced beauvericin: *F. acuminatum* var. *acuminatum*, *F. acuminatum* var. *armeniacum*, *F. anthophilum*, *F. avenaceum*, *F. beomiforme*, *F. dlamini*, *F. equiseti*, *F. longipes*, *F. nygamai*, *F. oxysporum*, *F. poae*, *F. sambucinum*, and *F. subglutinans* (Logrieco et al. 1998). The metabolite has also been found as a natural contaminant of maize in Italy, Austria, Poland, South Africa and the USA and was detected in all maize hybrids (Pascale et al. 2002). These results and other published data (Munkvold et al. 1998; Fotso et al. 2002; Logrieco et al. 2002; Moretti et al. 2002) confirm that beauvericin is a common metabolite of many phytopathogenic *Fusarium* species and occurs in diverse foods and feeds contaminated with *Fusarium* species.

Investigations of beauvericin have demonstrated that this metabolite has insectidal, antibiotic, cytotoxic, and ionophoric properties. According to Roberts (1981), some toxic effects have been noticed against bacteria, mosquito larvae, brine shrimp and adult houseflies, but not against silkworm larvae at 1000 ppm in artificial diet. Recently, Ganassi et al. (2002) reported some effects of beauvericin on the aphid *Schizaphis graminum*. Antimycobacterial (*Mycobacterium tuberculosis*) and antiplasmodial (*Plasmodium falciparum*) activity of beauvericin and beauvericin A isolated from *P. tenuipes* BCC 1614 was reported by Nilanonta et al. (2000).

Beauvericin is a specific cholesterol acyltransferase inhibitor and is toxic towards *Artemia salina* larvae and against insect, murine and human cell lines. It can induce programmed cell death similar to apoptosis and causes cytolysis (Logrieco et al. 1998; Vey et al. 2001; Pascale et al. 2002). Investigations on the effect of beauvericin to the insect cell line SF-9 from the lepidopteran *Spodoptera frugiperda* revealed a clear cytotoxicity (Calo et al. 2003; Fornelli et al. 2004). One micromolar concentration of beauvericin caused about 10% decrease in the number of viable cells, and the effect increased at higher concentrations. However, in time-course experiments, no effect of beauvericin at 30 μM was noticed on cell viability (Calo et al. 2003). Cytotoxicity of beauvericin to two human cell lines of myeloid origin was reported by Calo et al. (2004). After an exposure time of 24 h, a decline in viability of cells was observed at a concentration of 10 μM or higher. In turkeys, fumonisin B-1 and beauvericin may affect the immune functions by suppressing proliferation and inducing apoptosis of peripheral blood lymphocytes (Dombrink-Kurtzman 2003).

Beauvericin did not cause any symptoms on roots of melon, tomato, wheat and barley, however, it showed high toxicity towards protoplasts of these plants (see Moretti et al. 2002).

With respect to the natural occurrence of beauvericin in *Fusarium*-contaminated food, feeding trials were conducted with broilers to study the effect of diets containing the mycotoxins moniliformin and beauvericin from natural contamination in the field (Zollitsch et al. 2003). The results indicate that dosages of up to 2.7 mg moniliformin and 12 mg beauvericin per kg diet showed no effect on any of the traits observed.

Whether beauvericin should be seen as a food mycotoxin is not yet clear, because it mostly co-occurrs with other *Fusarium* metabolites, such as fumonisins. In any case, it is likely that beauvericin found and isolated from foods and feeds in nature derives from *Fusarium* species rather than from *B. bassiana*.

Bassianin and tenellin are two yellow-coloured non-peptide secondary metabolites which inhibit the erythrocyte membrane ATPases (Jeffs & Khatchatourians 1997). There is very little published information on these two metabolites (see Strasser et al. 2000).

Bassianolide is another cyclo-octadepsipeptide produced by *B. bassiana* with ionophoric and antibiotic activity similar to beauvericin (see Strasser et al. 2000; Vey et al. 2001). The biosynthesis of a structurally related substance, called PF1022, was reported by Weckwerth et al. (2000). PF1022 has strong anthelmintic properties and was found in a fungus producing only sterile mycelium.

Bassiacridin is a toxic protein, that was purified from a strain of *B. bassiana* infecting locusts (Queseda-Moraga & Vey 2004). Injection of fourth instar nymphs of *Locusta migratoria* with the pure protein at relatively low dosage (3.3 μg toxin/g body weight) caused nearly 50% mortality. This insecticidal protein showed specific activity against locusts and has a limited similarity to a chitin binding protein from yeasts.

Beauveriolides and beauverolides are peptides with a similar structure to beauvericin and bassianolide (Namatame et al. 1999, 2004). Beauveriolides seem to have potential as drugs in human medicine. Beauveriolides I and III isolated from culture broth of *Beauveria* sp. (FO-6979) showed potent inhibitory activity of lipid droplet accumulation in primary mouse peritoneal macrophages. They are the first microbial cyclodepsipeptides with demonstrated *in vivo* antiartherosclerotic effects and show promise as potential lead compounds as antiartherosclerotic agents (Namatame et al. 2004). Recently, a patent for production of beauveriolide I or III by *Beauveria* sp. (FO-6979) on selective media was granted (Omura & Tomoda 2005).

Oosporein is the major secondary metabolite produced by *B. brongniartii* and is also produced by many isolates of *B. bassiana*. Comprehensive overviews on oosporein are presented by Strasser et al. (2000), Vey et al. (2001) and Seger et al. (2005a,b). This red-coloured pigment is a dihydroxybenzoquinone, which is also produced by many soil fungi. It is an antiviral compound and has antibiotic activity against gram-positive bacteria, but little effect on gram-negative bacteria. Obviously, oosporein has no antifungal and phytotoxic effects. It has been reported to cause avian gout in broiler chicks and turkeys and was found to be toxic to 1-day-old chicks. Furthermore, studies on its toxicity in mice and hamsters indicated an LD_{50} value of 0.5 mg kg^{-1} body weight after intraperitoneal injection (Manning & Wyatt 1984; Vey et al. 2001). *In vivo* and *in vitro* studies on the distribution of oosporein in the environment revealed negligible amounts present. The maximum amount of oosporein produced in a culture medium was 300 mg L^{-1}, 3.2 mg kg^{-1} in the commercial product 'Melocont®-Pilzgerste' (Agrifutur-Kwizda), 200 μg in a mycosed larva, 0.02 mg m^{-2} in soil enriched with the commercial product and 6.4 mg m^{-2} in soil enriched by mycosed larvae (Strasser et al. 2000). These results demonstrate that the quantity of oosporein produced by these fungi *in vivo* is usually much less than that secreted in nutrient rich liquid media. No fungal metabolites, such as oosporein, were detected in potato plants and tubers after application of 'Melocont®-Pilzgerste' into the soil of a potato field (Abendstein et al. 2000; Strasser et al. 2000; Seger et al. 2005c).

Oxalic acid is secreted by *B. bassiana* and *B. brongniartii* (Müller-Kögler 1965; Roberts 1981). It is considered an important pathogenicity determinant because it can solubilise specific cuticular proteins (see Vey et al. 2001). In the grasshopper *Melanoplus sanguinipes*, a synergistic activity between oxalic acid and *B. bassiana* conidia was observed (Bidochka & Khachatourians 1991), however the acid was not related to virulence in grasshoppers (Bidochka & Khachatourians 1993). Cell-free culture supernatants of *B. bassiana* containing oxalic acid caused mortality in several tick species after dipping (Kirkland et al. 2005), which support the hypothesis that oxalic acid secretion by *B. bassiana*, coupled to a reduction in the pH of the medium, acts as a potent acaricidal factor during pathogenesis.

Effect of environmental factors (temperature, humidity, solar radiation)

The propagation and survival of any microorganism in the environment is strongly affected by several abiotic and biotic factors. The most important abiotic environmental constraints for fungi are temperature, humidity or moisture and solar radiation. These factors are also responsible for effective, commercial use of entomopathogenic fungi. The importance of these ecological parameters was recognised early (Clerk & Madelin 1965; Müller-Kögler 1965; Roberts & Campbell 1977; Keller & Zimmermann 1989; Fuxa 1995). This section summarises some general observations of the effect of temperature, humidity and solar radiation on the activity and longevity of *B. bassiana* and *B. brongniartii* in the laboratory as well as in the field.

Temperature. Temperature can affect an entomopathogen in different ways by influencing the germination, growth and viability of the fungus on and in the host insect and in the environment. High temperatures may inactivate an entomopathogen before contact with the pest insect or may reduce or accelerate the growth within an insect depending on the temperature requirements of the entomopathogen and the host insect. In contrast, low temperatures may reduce or stop the germination and growth, thus impair or prolong a successful infection, e.g. against soil dwelling pest insects.

In *B. bassiana,* the optimum temperature is 23–28°C, the minimum 5–10°C and the maximum about 30–38°C, depending on the isolates tested (Müller-Kögler 1965; Roberts & Campbell 1977). These values have been substantiated later by other scientists (e.g. Hywel-Jones & Gillespie 1990; Fargues et al. 1997; Hallsworth & Magan 1999). Among African isolates of *B. bassiana*, germination, radial growth and sporulation of all isolates were retarded at 15 and 35°C, while the optimum temperature of different isolates was between 20 and 30°C (Tefera & Pringle 2003) or 25–30°C (Ekesi et al. 1999). In contrast, *B. bassiana* isolates from subantarctic soils of Macquarie Island germinated at 5°C (Roddam & Rath 1997). The thermal death point of *B. bassiana* spores is at 50°C for 10 min (Walstad et al. 1970). In *B. brongniartii* the temperature range for growth and sporulation is between 2 and 33°C with an optimum of 22–23°C (Müller-Kögler 1965; Roberts & Campbell 1977). Growth of *B. brongniartii* has been noticed during storage at 2°C (Aregger 1992).

Humidity. Humidity is a very important environmental factor affecting the efficacy and survival of entomopathogens. Spore germination on the insect cuticle and sporulation

after outgrowth of the dead host insect require high moisture. On the other hand, high or low humidity in conjunction with high temperature may affect the viability and persistence of fungal spores. We have to distinguish between the macroclimate or macrohumidity and the microclimate or microhumidity on leaf or insect surfaces when considering moisture effects in the field. Generally, the range of relative humidity (RH) for germination of *B. bassiana* conidia is 100–92% (Walstad et al. 1970; Hallsworth & Magan 1999). A slight reduction in germination occurs at 99% RH, while germination and growth is retarded at 94% and 92%. However, fungal infections of insects have been noticed at relatively low macrohumidities of 60–70%. Presumably the microhumidity at the surface of the host integument or foliage was higher in these cases. Successful infection at low relative humidities has also been observed with oil formulations (Prior et al. 1988; Bateman et al. 1993; Vidal et al. 2003). Obviously, fungal spores are able to germinate and infect the insect when covered by a thin oil layer on the insect cuticle independent of the surrounding relative humidity. The longevity of conidia at different temperatures is also strongly affected by the relative humidity and moisture content of the conidia. For example, the viability of dry conidia of *B. bassiana* was 635 days at 8°C and 0% RH in contrast to 28–56 days at 25°C and 75.8% RH. A lower RH increases the longevity of spores even at higher temperature (Clerk & Madelin 1965).

Solar radiation. Sunlight, especially UV-B (290–330 nm) and UV-A (330–400 nm), is the most detrimental environmental factor affecting the field persistence of fungal insecticides. The results presented in the literature reveal that entomopathogens are inactivated within hours or days when exposed to sunlight (Gardner et al. 1977). In laboratory experiments under simulated sunlight, 99% of all *B. bassiana* conidia were inactivated at UV-C after nearly 16 min, and at UV-A and UV-B after about 31 min (Krieg et al. 1981). After irradiation with simulated sunlight, Ignoffo and Garcia (1992) found a half life of *B. bassiana* conidia of about 2 h. The influence of simulated sunlight (295–1100 nm) on the survival of conidia of 65 isolates of *B. bassiana* demonstrated that the survival decreased with increasing exposure, i.e. an exposure for 2 h or more was detrimental to all isolates tested (Fargues et al. 1996). In the laboratory, the survival of conidia applied in water onto glass coverslips and on crested wheatgrass (*Agropyron cristatum*) was reduced by greater than 95% after 15 min exposure to UV-B radiation (Inglis et al. 1995). Conidial survival in oil was more pronounced on glass (74% mortality after 60 min) than on leaves (97% mortality after 60 min). Significant differences in susceptibility to simulated sunlight among isolates of *B. bassiana* were demonstrated by Morley-Davies et al. (1995). Also, diffuse sunlight has inactivating abilities as demonstrated in the entomopathogenic fungus *Paecilomyces fumosoroseus* (Smits et al. 1996).

The detrimental effects of sunlight implicating the relatively short persistence of microbial control agents after application have led to incorporation of various UV-protectants to conidial formulations (e.g. Inglis et al. 1995; Edgington et al. 2000; Cohen et al. 2001; Leland & Behle 2005).

Analytical methods to determine and quantify residues

There are several methods and techniques for selective isolation of entomopathogenic fungi, including *B. bassiana* and *B. brongniartii* (Goettel & Inglis 1997). As larvae of

Galleria mellonella are notoriously sensitive to entomopathogenic fungi, the 'Galleria bait method' (Zimmermann 1986) is generally used for qualitative analysis and to indirectly isolate fungi from soil. The use of selective media in combination with the soil serial dilution plating method or a leaf washing technique gives quantitative results. Different selective media for reisolation of *B. bassiana* and *B. brongniartii* from soil or plants have been used (Veen & Ferron 1966; Müller-Kögler & Stein 1970; Joussier & Catroux 1976; Doberski & Tribe 1980; Beilharz et al. 1982; Chase et al. 1986; Strasser et al. 1996). As selective agents, the first media contained oxgall, rose Bengal, crystal violet, cycloheximide and antibiotics, such as streptomycin, chloramphenicol and/or tetracycline. In 1982, Beilharz et al. found, that media based on oatmeal agar containing the fungicide Dodine® (*N*-dodecylguanidine monoacetate) considerably improved the selectivity for *B. bassiana* and *M. anisopliae*. Later, the addition of benomyl was suggested by Chase et al. (1986). For *B. brongniartii*, Strasser et al. (1996) demonstrated that a nutrient medium containing cycloheximide (0.05%), Dodine® (0.1%) and antibiotics was best suited for selective isolation of this fungus from soil. In all cases, the minimum number for recovery of both *Beauveria* species is about 10^2 conidia or propagules per 1 g of soil.

During a study on the microbial flora in heavy metal polluted soils, Bååth (1991) found that entomogenous fungi were highly tolerant to copper. Therefore, a copper-based medium could also be useful for selective isolation of these fungi.

A variety of different biochemical and molecular methods has been developed to identify and distinguish among strains of entomopathogenic fungi (see Bidochka 2001; Rehner & Buckley 2005). Neuveglise et al. (1997) found that 28s rDNA group-I introns are a powerful tool for identifying strains of *B. brongniartii.* Also, strain-specific microsatellite markers in *B. brongniartii* (Enkerli et al. 2001) and in *B. bassiana* (Rehner & Buckley 2003) have been identified. These microsatellite markers can also be applied to study fungus populations and to monitor the fate of specific strains in the environment (Enkerli et al. 2004).

Fate and behaviour in the environment

Studies on the fate and behaviour of a microbial control agent are important with respect to its ecological safety, e.g. potential unintended effects on non-target organisms, including their displacement, unintended distribution in the environment or contamination of groundwater.

Mobility and persistence in air

Conidia of *B. bassiana* and *B. brongniartii* are dry, of small size and are produced in powdery clusters. Therefore, it is obvious that these types of conidia are easily transported by air. However, there are only few observations of *Beauveria* as an airborne fungus, but these results document that *Beauveria* spp., especially *B. bassiana*, do occur naturally in the air (Airaudi & Filipello-Marchisio 1996; Rainer et al. 2000; Shimazu et al. 2002; Lackner et al. 2004; Nolard 2004; see *Natural occurrence and geographical distribution* and *Effects on mammals and human health*).

The transportation of fungal spores through the air by insects has been documented in many cases. Insects may function as a mechanical carrier or vector for *B. bassiana*, e.g. in order to transmit the fungus from a contamination device into the pest

population (for references see Kreutz et al. 2004). For example, sap beetles were contaminated with *B. bassiana* in an autoinoculative device and transferred the fungus to overwintering sites (Dowd & Vega 2003). The species was also isolated from free flying sap beetles caught in traps. Similar transmission of *B. bassiana* in laboratory and semi-field experiments has been demonstrated with the bark beetle, *Ips typographus* (Kreutz et al. 2004). *Carpophilus freemani* is a fungivore that is frequently found in European corn borer, *Ostrinia nubilalis*, tunnels in corn. Beetles fed *B. bassiana* excreted viable conidia in 14% of their fecal droppings and, thus, may transfer the fungus both via their fecal droppings and mechanically (Bruck & Lewis 2002a).

The viability of *B. bassiana* spores in the air is mainly affected by temperature, humidity and sunlight (see *Effect of environmental factors* (*temperature, humidity, solar radiation*)). The longevity of dry conidia on glass surfaces decreases as the storage temperature increases from 8 to 25°C and by exposure to light. High storage humidities reduce the germination sooner than low humidities (Clerk & Madelin 1965). Undoubtedly, natural sunlight, i.e. the UV-B (290–330 nm) and UV-A (330–400 nm) component, is one of the most important factors affecting the survival of fungal spores in the air and on plants. A rapid inactivation of *B. bassiana* conidia by ultraviolet radiation within hours has been demonstrated in the laboratory (e.g. Krieg et al. 1981; Fargues et al. 1996). Studies on the persistence of *B. bassiana* conidia on plant surfaces, such as soybean foliage, have demonstrated that one-half of the original activity was lost between 5 and 10 days post application (Gardner et al. 1977).

Viability on the plant surface may also be influenced by the plant type. Field evaluations of *B. bassiana* revealed a conidia persistence and infectivity up to 26 days on foliage of lettuce and celery. However, the number of colony forming units (CFUs) recovered on lettuce was significantly higher than on celery leaves (Kouassi et al. 2003). The viability of conidia of *B. bassiana* on phylloplanes of alfalfa (*Medicago sativa*) and crested wheatgrass (*Agropyron cristatum*) was reduced after four days by more than 75%, at 16 days more than 99% of the conidia on wheatgrass leaves and 28–85% on alfalfa leaves were destroyed (Inglis et al. 1993).

Mobility and persistence in water

There are different aspects on the mobility and persistence of fungal spores in water: (1) water can be used for longterm storage of fungi under laboratory conditions, (2) water is responsible for migration/percolation of spores into the soil, and (3) water as raindrops is responsible for dispersal.

Storage of fungal cultures in water is an easy and cheap method, which was used more than 60 years ago by Castellani (1939). Boesewinkel (1976) was able to store 650 plant pathogenic and saprophytic fungi successfully in sterile, distilled water at room temperature for periods up to seven years. Several entomopathogenic fungi were viable after storage in sterile aqueous solutions of 0.675% NaCl at 4°C for 2–3 years (Müller-Kögler & Zimmermann 1980).

Water is responsible for percolation of spores into soil and will be discussed in *Mobility and persistence in soil*. Rainfall plays an active role in the dispersal of *B. bassiana* from the soil to the surface of whorl-stage corn. Increased levels of crop residues reduce the amount of fungal transfer to the surface of young maize (Bruck & Lewis 2002b). Recently, both *Beauveria* spp. were found in surface water-derived drinking water in Norway (Hageskal et al. 2006).

Mobility and persistence in soil

The mobility of fungal spores in the soil is mainly due to water/rain and soil arthropods. In contrast, persistence depends on several abiotic and biotic factors. These are specific soil properties, temperature, moisture and water, and agrochemicals as abiotic factors and soil microorganisms as well as soil arthropods as biotic factors (Keller & Zimmermann 1989).

Mobility. The mobility of fungal spores in and into the soil is of relevance for two different reasons: (1) for effective biocontrol of soil dwelling pests, which mostly feed on roots of their host plants. In this case, the fungus has to be introduced into the root area in order to come into contact with the host insect; (2) for environmental safety considerations, i.e. it has to be demonstrated that the fungus only percolates to the root area and does not reach or contaminate the ground water. In this respect, Marshall and Bitton (1980) pointed out that microbial adhesion is of fundamental significance in the function and interaction between microorganisms, i.e. attachment of microorganisms to surfaces ensures that they are not eliminated from the particular ecosystem. This means that attachment may be a prerequisite for the relationship between micro- and macroorganisms.

Concerning *B. bassiana* and *B. brongniartii,* it has been proven that these fungi occur naturally in the soil throughout the world (see *Natural occurrrence and geographical distribution*). It has further been demonstrated that *B. bassiana* could be found from 0–5 to 20–25 cm depth in different arable soils (Mietkiewski et al. 1995). The question however is, how deep can these fungi percolate into the soil. First experiments with other fungal species show that the spore and soil type may affect the migration. Spores of *Zygorrhynchus* and *Gliomastix* wash readily through a sand column up to 30–40 cm, whereas *Penicillium* spores show very little movement (5–10 cm) (Burges 1950). Spores that have a mucilaginous coat wash down readily, while spores which have a waxy non-wetting coat remain on the surface (Burges 1950). According to Hepple (1960), water is responsible for vertical movement of spores of *Mucor ramannianus*, but only over short distances. About 75% of conidia of the entomopathogenic fungus *Nomuraea rileyi* layered on a 10.5-cm column of sand were recovered in the filtrate after exposure to the equivalent of 16.25 cm of rain (Ignoffo et al. 1977). However, no conidia were recovered in filtrate from a silt-loam soil and over 90% of the recovered conidia were in the upper 2 cm of the column. The spores had probably been adsorbed on clay or organic particles. Studies on the vertical movement of wet and dry spores of *M. anisopliae* through a 30-cm sand column revealed that less than one spore per 1 mL effluent was found (Zimmermann 1992). The effect of percolating water on spore movement through soil was also studied using a plant-pathogenic isolate of *Fusarium oxysporum* f. sp. *niveum*. Formulations were placed on soil columns and artificial rain was applied. In general, 10-fold fewer CFU were recorded at an 8–10 cm depth compared with a 0–2 cm depth (Gracia-Garza & Fravel 1998).

Vertical movement of commercially formulated conidia of *B. bassiana* was measured in four, sifted soil types in 30.6 cm columns (Storey & Gardner 1987). When applied as an aqueous suspension to the soil, >90% of the viable CFU's were recovered in the upper 15.2 cm in two soil types. Approximately 12.5% of the CFU's moved through the 30.6 cm column of the two other types and were collected in the effluent. When the vertical movement of formulated *B. bassiana* conidia was investigated in

undisturbed soil types, the migration was considerably less than that observed in columns of sifted soil. The majority, i.e. >94% of the conidia remained in the upper 5 cm of all four soil profiles (Storey & Gardner 1988; Storey et al. 1989). These observations indicate that the soil type, the soil pore structure (sifted versus undisturbed soil) and probably also the shape and size of spores affect their movement through the soil. Finally, movement in horizontal and vertical direction is also possible by Collembola (Dromph 2003) and earthworms (Hozzank et al. 2003).

Persistence. As already mentioned, persistence of fungal spores in the soil is affected by several biotic and abiotic factors. In this respect, one main aspect is soil fungistasis. The term describes the phenomenon, whereby (a) viable fungal propagules, not under the influence of endogenous or constitutive dormancy, do not germinate in non-sterile soil at temperature and moisture favourable for germination, or (b) growth of fungal hyphae is retarded or terminated by conditions of the soil environment other than temperature and moisture (Watson & Ford 1972). A widespread fungistasis in soils was found and postulated about 50 years ago (Dobbs & Hinson 1953). Soil fungistasis has been shown to be general in natural soils and to be a dynamic phenomenon.

First experiments concerning the survival of *B. bassiana* in the soil were carried out by Huber (1958) and later by Wartenberg and Freund (1961). The authors found that antibiotic microorganisms suppress the germination of conidia of *B. bassiana* in soil. They concluded that *B. bassiana* is a weak saprophyte and speak of a 'conservation effect' induced by antibiotic microorganisms, such as Actinomycetes. According to Clerk (1969), several authors have reported that conidia of *B. bassiana* are subject to fungistatic effects in natural soils. However, the nature of the inhibitor(s) responsible for soil fungistasis is still unknown, although several authors consider that inhibitory substances released by soil microorganisms play a major role in fungistasis. Clerk (1969) found that conidia of *B. bassiana* are able to germinate in sterilised soil or in soil stimulated by an external source of nutrients, i.e. the presence of insects in the soil might influence the behaviour of conidia. Conidial germination and hyphal growth of *B. bassiana* was inhibited in unsterilised aqueous extracts of soil. Extracts of the deepest soil layer were less inhibiting than extracts of overlaying layers of humus-rich soil. The inhibition was reduced by autoclaving and by filtering the extracts, indicating that the soil microbiota has an impact on the activity of *Beauveria* spp. When *B. bassiana* and *B. brongniartii* were stored at 4°C in sterile soil for preservation purposes, Müller-Kögler and Zimmermann (1980) found that the *B. bassiana* isolate was still viable after six years, the *B. brongniartii* isolate after four years with decreased viability after six years.

According to Lingg and Donaldson (1981), the viability of *B. bassiana* conidia in soil was primarily dependent on temperature and soil water content. Conidia half-lives ranged from 14 days at 25°C and 75% water saturation to 276 days at 10°C and 25% water saturation. Conidia held at −15°C exhibited little or no loss in viability. Conidia were not recoverable after 10 days from soils at 55°C. Conidia survival in nonsterile soil amended with carbon (wheat and pea straw, gluciose, chitin) and/or nitrogen (KNO_3, NH_4Cl, urea, ammonium tartrate) sources was greatly decreased with often complete loss in less than 22 days, whereas conidia in sterile soil treated in the same manner showed dramatic increase. The fungistatic effect in amended nonsterile soil was possibly related to *Penicillium urticae*, which was often isolated and which produced a water-soluble inhibitor of *B. bassiana*. Groden and Lockwood

(1991) noticed a fungistatic effect on *B. bassiana* in two soils from different areas. Fungistatic levels varied between years and increased with increasing pH in soil. A loss of fungistatic mechanisms by sterilisation was postulated by McDowell et al. (1990). Sterilising soil before bioassays resulted in a 10- and 1000-fold reduction in LC_{50} values required to kill first and third instars of *Elasmopalpus lignosellus* (Lepidoptera: Pyralidae), respectively. These findings are also supported by Rosin et al. (1996) who found that soil containing fresh manure was detrimental to *B. bassiana*, whereas high rates of composted manure were beneficial. Obviously, certain factors in the fresh manure reduce the survival of *B. bassiana*.

The persistence of *B. bassiana* conidia in artificially contaminated soil was investigated under laboratory and field conditions (Müller-Kögler & Zimmermann 1986). When starting the experiment in October, the number of viable conidia decreased from about 10^6 g^{-1} dry soil to about 10^4 or 10^3 after one year according to the soil depth. When the experiment was started in May, the corresponding values were 10^7 at the beginning to about 10^5 conidia g^{-1} dry soil after 1 year. Storey et al. (1989) estimated the persistence of applied conidia to be about 200 days, while the granular formulation of conidia persisted for a longer period. The persistence of *B. bassiana* blastospores in soil and their protection by clay-coating was investigated by Fargues et al. (1983). Naked blastospores of *B. bassiana* were inactivated after 3 weeks incubation in soil, while clay-coated blastospores were still active after two months at 20°C. Clay coating is a protection against biodegradation of fungal propagules by soil bacteria and protozoa. The authors stated that antagonists implicated in lysis of blastospores must be considered an important part of the environmental response to a massive introduction of a fungus for biocontrol. Studdert et al. (1990) studied the relationship between soil water potential and temperature on survival of *B. bassiana* clay coated and noncoated conidia in two nonsterile soils. The longest mean half-life value was 44.4 weeks for conidia in sandy loam at −10 bars (0.0 bars = saturation) and 10°C. Clay-coating increase the survival of conidia. Survival was longer in the low organic soil compared to the high organic peat. The results suggest that conidia survival is affected by several physical factors and the soil microbiota. With an experimental biodegradation method, Fargues and Robert (1985) found that inocula of *B. bassiana* were substantially degraded and subject to 70–80% dry weight loss after six months at 19°C. In another experiment, conidia were spread on test areas as water suspensions at a rate of 10^{10} spores m^{-2}. After one year, the mean counts were about only 0.2% for *B. bassiana* of the originally spread spore amount. In loamy soil, most of the spores were found at 0–5 cm, while in humus, they were found in deeper soil layers at 5–15 and 15–20 cm (Tyni-Juslin & Vänninen 1990). Inglis et al. (1997) investigated the influence of three formulations (water, oil, and 15% oil emulsion) and two crops (alfalfa and crested wheatgrass) on the deposition and subsequent persistence of *B. bassiana* conidia in soil. Reductions during winter after 225–272 days were less than one order of magnitude. Neither crop nor formulation influenced conidial persistence in a clay–loam soil.

Investigations on the persistence of *B. brongniartii* were done mainly in Switzerland within the frame of experiments for cockchafer control. Applications of *B. brongiartii* fungus kernels from May to August generally resulted in an increase of $1–5 \times 10^3$ CFU g^{-1} dry soil compared to untreated control plots (Kessler et al. 2003). Soils treated in October and November yielded no increase. Soil temperatures between 20 and 25°C and a high clay content had a positive effect on the occurrence and density

of *B. brongniartii*, whereas temperatures above 27°C had a negative influence. The survival of *B. brongniartii* in soil was further examined for over 16 months after application of fungus kernels in different soil types in Switzerland (Kessler et al. 2004). In the absence of the host insect, *M. melolontha*, the reduction in the CFU in soil was nearly 90%. In soils with high organic content, the decline was more pronounced. When grubs of *M. melolontha* were present, the survival was significantly longer. The rapid decrease of the fungus in soil without the host reveals the high specificity of the fungus and that a saprophytic multiplication is unlikely (Längle et al. 2005).

Monitoring of introduced microorganisms in the environment is essential not only for the development of new biocontrol agents, but also for understanding of their interactions with the living environment, their ecological impact and safety assessments. Several years after *B. brongniartii* was applied against *M. melolontha* in various field tests in Switzerland, isolates were recovered from soil and subjected to genetic analyses using specific microsatellite markers (Enkerli et al. 2004). The applied *B. brongniartii* strains were detected at all sites up to 14 years after their application. In addition to the applied strains, indigenous and mutated or intermediate strains were also isolated. The results suggested that applied and indigenous strains of *B. brongniartii* could coexist in the same habitat. These observations are supported by Castrillo et al. (2004), who observed a large number of vegetative compatibility groups (VCG) among strains of *B. bassiana* and a very low level of recombination which may be a barrier preventing genetic exchange between dissimilar strains in the field.

Effects on non-target organisms

Effects on other microorganisms

Investigations on the natural prevalence of *B. bassiana* have shown that this fungus widely occurs in the soil as well as on insects in the aerial environment. This means that there is a long lasting evolutionary coexistence with other microorganisms that includes different forms of interactions.

From the viewpoint of safety, the main concern is that microorganisms applied for biological control could potentially pre-empt or displace other nontarget microorganisms. After application on plants or in the soil, biocontrol agents should be able to survive and maintain themselves for biocontrol activity, but they should not interfere with the resident microbiota. For example, studies on inundative release of an atoxigenic strain of *Aspergillus flavus* into the soil of cotton fields showed that the native, toxigenic isolates were almost completely displaced (Cotty 1994). Yet, in a risk analysis case study using *Fusarium* species, the effect of antagonistic *Fusarium oxysporum* to control *Fusarium* wilts on the resident soil microbiota revealed that the introduction of wild-type and genetically manipulated antagonistic strains of *Fusarium oxysporum,* released alone or in mixture, did not interfere with the microbial equilibrium of a natural soil (Gullino et al. 1995). Similar results were also obtained by Wang et al. (2004) in *B. bassiana* and Enkerli et al. (2004) in *B. brongniartii*. Wang et al. (2004) monitored the fate of inundatively applied strains of *B. bassiana* against *Dendrolimus punctatus* in southwest China. During one year, the indigenous and exotic strains were reisolated, but the indigenous strains predominated in the local environment, indicating that they were not displaced by the exotic ones. Enkerli et al. (2004) studied the behaviour of introduced *B. brongniartii* strains for control of *M. melolontha* grubs in

Switzerland. The results suggested that applied and indigenous strains of *B. brongniartii* could coexist in the same habitat. Furthermore, in *B. bassiana*, a genetic exchange between indigenous and introduced strains in the field is unlikely due to the large number of vegetative compatibility groups (Castrillo et al. 2004).

There are several reports on interactions of *Beauveria* spp. with hyperparasitic, antagonistic and especially, phytopathogenic fungi. A hyperparasitic fungus attacking *B. bassiana* and *B. brongniartii* is the ascomycete *Syspastospora parasitica*, formerly known as *Melanospora parasitica* (Müller-Kögler 1961; Markova 1991; Posada et al. 2004). Lingg and Donaldson (1981) reported that the survival of conidia of *B. bassiana* in nonsterile soil amended with carbon sources, nitrogen sources or combinations of both was possibly related to *Penicillium urticae*, which produced a water-soluble inhibitor of *B. bassiana*. There are also various interactions with *Clonostachys* spp. and *Trichoderma* spp. which may suppress or overgrow *B. bassiana in vitro* (Krauss et al. 2004; Zimmermann unpublished). Meanwhile, there is an increased interest to test and use *B. bassiana* also against plant pathogens (Ownley et al. 2004). According to the experiments, *B. bassiana* isolate 11–98 could reduce *Rhizoctonia solani* damping-off of tomato in greenhouse tests, and also protect cotton against a seedling disease complex in some sites. Laine and Nuorteva (1970) had previously observed that *B. bassiana* and *B. brongniartii* (*B. tenella*) had a strong antagonistic effect on *Fomes* (*Heterobasidium*) *annosus*. *Beauveria bassiana* had also an antagonistic activity against *Ophiostoma ulmi* (*Ceratocystis ulmi*) (Gemma et al. 1984) and significantly reduced the disease incidence of *Phoma betae*, the blackleg of beet (Roberti et al. 1993). Under greenhouse conditions, *B. bassiana* and *B. brongniartii* were antagonistic to *Pythium ultimum*, *P. debaryanum* and *Septoria* (*Leptosphaeria*) *nodorum*, while *Pythium irregulare*, *Phoma betae*, *Phoma exigua* var. *foveata* and *Rhizoctonia solani* showed resistance to both *Beauveria* species (Vesely & Koubova 1994). The mycelial growth of three phytopathogens of the genera *Fusarium*, *Armillaria* und *Rosellinia* was significantly reduced by filtrates of *B. bassiana* (Reisenzein & Tiefenbrunner 1997), and metabolites from *B. bassiana* produced in liquid culture inhibited the growth of several *Fusarium* spp. (Langbauer et al. 1996). Mycelial growth and conidial germination of *Botrytis cinerea* and *Fusarium oxysporum* were inhibited by a culture filtrate of *B. bassiana* (Bark et al. 1996).

Effects on plants

Both *Beauveria* species are typical soil dwelling fungi and are known to be entomopathogens. Nevertheless, the question of possible phytopathogenic side-effects or any other interactions with plants should be raised. In the past 100 years, *B. bassiana* and *B. brongniartii* have been used for biocontrol of so many leaf- and root-feeding pest insects, that there was ample opportunity of observation of detrimental effects of these fungi in plants. In summarising the past literature, Müller-Kögler (1965) concluded that side-effects or any phytopathogenic activity on plants are not known. We can presently come to the same conclusion. The natural occurrence on plants is reviewed in *Natural occurrence and geographical distribution*.

Recent research has demonstrated that there are various tri-trophic interactions between the plant, the pest insect feeding on the plant and entomopathogenic fungi attacking these herbivores. Elliot et al. (2000) hypothesise whether plants use

entomopathogens as bodyguards against herbivores. The most interesting interactions are as follows:

1. Plants may affect the infection by the entomopathogen;
2. Plants may affect the persistence of the entomopathogen;
3. *B. bassiana* can persist as an endophyte within plants.

There are several reports indicating that the plant species may affect the infectivity and persistence of *B. bassiana*. Ramoska and Todd (1985) found that chinch bugs, *Blissus leucopterus*, fed on sorghum and corn were more resistant to *B. bassiana* than those on barley, and *Bemisia argentifolii* reared on cotton was significantly less susceptible to the fungus than white flies reared on melon (Poprawski & Walker 2000). On foliage of lettuce and celery, the mortality of *Lygus lineolaris* adults seven days post treatment was 91 and 78%, respectively (Kouassi et al. 2003). Nymphs of *Trialeurodes vaporariorum* were highly susceptible to *B. bassiana* on cucumber plants, while insects reared on tomato plants were significantly less susceptible (Poprawski et al. 2000). It is assumed that sequestered tomatine by *T. vaporariorum* nymphs may explain the inhibition of *B. bassiana* after the penetration process, as tomatine was demonstrated *in vitro* to have a detrimental effect on *B. bassiana.*

Beauveria bassiana has been reported to be an endophyte of certain plants, especially corn (Bing & Lewis 1992). Studies have demonstrated that the fungus, applied to whorl-stage corn by foliar application or injection, colonised, translocated and persisted in corn plants. Some conidia of *B. bassiana* are able to germinate on the leaf surface of corn and penetrate it. Virulence bioassays demonstrated that *B. bassiana* does not lose virulence against *Ostrinia nubilalis* once it has colonised corn (Wagner & Lewis 2000). In the greenhouse, *B. bassiana* was applied as a liquid seed treatment to Bt transgenic corn hybrids and their near isolines (2×10^{10} conidia mL^{-1}), and no significant differences in seed germination or presence of root pathogens were observed (Lewis et al. 2001). Recent experiments revealed that *B. bassiana* may also function as an endophyte in cocoa seedlings (Posada & Vega 2005), in banana tissue culture plants (Dubois et al. 2005) and in opium poppy, *Papaver somniferum* (Quesada-Moraga et al. 2006).

Another aspect of the tri-trophic interactions between plants, entomopathogenic fungi and pest insects is the fact that toxic metabolites of *Beauveria* spp. may enter the plants. As already mentioned in *Production of metabolites/toxins*, beauvericin derived from *Fusarium* species and not from *B. bassiana* is obviously widespread in maize and food. Beauvericin did not cause any symptoms on roots of melon, tomato, wheat and barley, however, it showed high toxicity towards the protoplasts of these plants (see Moretti et al. 2002). The phytotoxic potential of *B. brongniartii* and its main metabolite oosporein were evaluated against seed potatoes (*Solanum tuberosum*) *in vitro* and *in situ*. The weight of haulm and tubers was unaffected by *B. brongniartii*, and no oosporein was detected in the potatoes. Therefore, the species pose no risks to potato plants or tubers (Abendstein et al. 2000; Seger et al. 2005a; See *Production of metabolites/toxins*).

Effects on soil organisms

As mentioned, *B. bassiana* and *B. brongniartii* are commonly found in soil. They generally have a broad host range and are often used for biocontrol of soil dwelling

pests. Therefore, possible interactions or effects on other non-target soil inhabiting invertebrates should be noticed.

In 1964, Samšiňák found that the mites *Tyrophagus putrescentiae* and *Acarus siro* are not susceptible to *B. bassiana*. *Tyrophagus putrescentiae* feeds on insects and also on dead, *Beauveria*-infected larvae. This mite species is also able to transmit spores of *B. bassiana* from fungus-infected larvae of *Galleria mellonella* to healthy ones. *Beauveria bassiana* was also found in high frequency on a great number of the collembolan *Onychiurus subtenuis* (Visser et al. 1987). The authors concluded that there was no indication that *B. bassiana* killed the collembolan. The collembolan *Folsomia candida* was not susceptible to *B. bassiana*. It consumed and inactivated the insect pathogen without causing mortality or any other harmful effects (Broza et al. 2001). Pathogenicity tests of *B. bassiana* and *B. brongniartii* were conducted against adults of *Folsomia fimetaria*, *Hypogastrura assimilis* and *Proisotoma minuta* (Dromph & Vestergaard 2002). By dipping the collembolans in 1×10^7 conidia mL^{-1} suspension and in one case also in 1×10^8 conidia mL^{-1}, none of the fungal isolates increased mortality over the controls. After continuous exposure of *F. fimetaria* and *P. minuta* to conidia of *B. brongniartii* for 14 days at 20°C in sphagnum containing 1×10^8 conidia g^{-1} wet weight, one of the *B. brongniartii* isolates increased the mortality significantly. In a test of the attractiveness of these fungi for the three collembolan species, *B. brongniartii* was found to be more attractive than baker's yeast. Mites were also observed feeding on *B. brongniartii* killed white grubs of *Melolontha* spp. without any sign of infection (Zimmermann, unpubl.).

Dispersal of entomopathogenic fungi by Collembolans has been demonstrated by several authors (Samšiňáková & Samšiňák 1970; Zimmermann & Bode 1983; Dromph 2001, 2003). For example, *B. bassiana* is distributed both in a horizontal and vertical direction by the mite *Sancassania phyllognathi* which is resistant to fungal infection (Samšiňáková & Samšiňák 1970). The transmission of spores of *B. bassiana* and *B. brongniartii* to a susceptible host, *Tenebrio molitor*, by the collembolans *F. fimetaria*, *H. assimilis* and *P. minuta* was also demonstrated (Dromph 2003).

These findings show that there are no or very low detrimental effects on the tested soil-dwelling collembolans and mites. In contrast, collembolans can act as vectors of *Beauveria* spp. and thus may play an important role for the dispersal and transmission of these fungi in soil.

Effects on aquatic organisms

No toxicity or pathogenicity was observed in *Daphnia magna* when exposed to 1×10^9 conidia of *B. bassiana* strain GHA per litre for 21 days (Goettel & Jaronski 1997). Strain GHA was also not infectious against the grass shrimp, *Palaemonetes pugio*, after percutaneous and oral contamination (Genthner et al. 1994b). In the mysid shrimp *Americamysis bahia* (formerly *Mysidopsis bahia*) *B. bassiana* conidia caused high mortalities, but these were attributed to a high particulate density since heat-killed controls also proved lethal (Genthner et al. 1994a).

Beauvericin has been found to be highly toxic towards *Artemia salina* larvae and murine cell lines and can induce apoptosis (Pascale et al. 2002). In the mysid *A. bahia*, beauvericin was toxic at an LC_{50} of 0.56 mg L^{-1} (Genthner et al. 1994a). To my knowledge there are no published studies regarding effects of other *Beauveria* metabolites as well as of *B. brongniartii* on aquatic organisms.

Effects on predators, parasitoids, honey bees, earthworms and other non-target arthropods

It is well known that *B. bassiana* has a wide host range, occurring on several hundred arthropod species; however, host specificity is really a strain-specific trait. For example, *B. bassiana* isolates from the lady beetle, *Olla v-nigrum*, were pathogenic to adult *O. v-nigrum* but not to adults of the Asian lady beetle, *Harmonia axyridis* (Cottrell & Shapiro-Ilan 2003). The GHA strain of *B. bassiana* was not significantly pathogenic to either *O. v-nigrum* or *H. axyridis*. In contrast, *B. brongniartii* has a much narrower host range being mostly restricted to members of the coleopteran family Scarabaeidae.

The practical use of these fungi in different crop protection systems raises the question of possible side-effects on non-target organisms. This is especially important when commercial products of these fungi are used on wide areas, e.g. for control of grasshoppers on meadows or of the European cockchafer species, or when *B. bassiana* is used together with beneficial insects in glasshouses.

Generally, there is a difference between the physiological host range and the ecological host range (Hajek & Butler 2000). The physiological host range demonstrates the range of insect species that can be infected in the laboratory, while the ecological host range demonstrates which insects can be infected in nature or under field conditions. Non-target insects which are infected under laboratory conditions, may not necessarily be infected in nature. This topic was also discussed in detail by Hajek and Goettel (2000) and Jaronski et al. (2003).

There are numerous papers on the effect of *B. bassiana* and *B. brongniartii* on beneficial and other non-target organisms. Examples are presented in Table V. Most of the studies were done in the laboratory and only a few in the field. One of the first comprehensive reports was given by Goettel et al (1990), who listed the effects of *B. bassiana* on nontarget invertebrates, such as bees and other pollinators, silkworms, predators and parasitoids. Further, general information is mentioned by Goettel et al. (1997, 2001) and Vestergaard et al. (2003). The last authors conclude that despite the wide host range of *B. bassiana*, evidence to date suggests that this fungus can be used with minimal impact on nontarget organisms, especially when isolate selection and spacio-temporal factors are taken into consideration. *Beauveria brongniartii* has a narrower host range, mainly including Scarabaeidae, and occurs worldwide in soil habitat. Laboratory bioassays demonstrated that it was possible to infect collembolans, cicindellid and carabid beetles unter stress conditions, while honey bees and earthworms were not affected (Table V). Data from field investigations did not reveal any indication of possible adverse effects on vertebrates, honeybees, beneficial insects, earthworms and plants (Vestergaard et al. 2003).

Effects on vertebrates (fish, amphibia, reptiles and birds)

Possible side-effects of entomopathogenic fungi on vertebrates and men were summarised by Müller-Kögler (1967) nearly 40 years ago. Today, detailed vertebrate safety tests are included in the registration process of commercial *Beauveria* products and have been conducted with several isolates of *B. bassiana*. Generally, both

Table V. Examples of effects of *B. bassiana* and *B. brongniartii* (strains and formulations) on beneficial and nontarget organisms.

Beneficial organism	Fungus (Strain/ Formulation)	Lab./ Field Trials (L/F)	Results/Observations	Reference
Amblyseius cucumeris	*B. bassiana* (Naturalis-L, BotaniGard WP)	L/F	No detrimental effect when sprayed onto excised cucumber leaves	Jacobson et al. (2001)
Aphidius colemani *Orius insidiosus* *Phytoseiulus persimilis* *Encarsia formosa*	*B. bassiana* (commercial formulation, strain JW-1)	L	Highly susceptible under laboratory conditions, lower infection rates in greenhouse	Ludwig and Oetting (2001)
Apis mellifera	*B. bassiana*	F	Conidia were applied in bee hives: low mortality and no noticeable effect on behaviour, larvae and colony characteristics	Alves et al. (1996)
Apis mellifera	*B. bassiana* (unformulated spore preparation)	L	*B. bassiana* reduced bee longevity at the two highest concentrations tested and caused mycosis at 10^6–10^8 spores per bee	Vandenberg (1990)
Apis mellifera	*B. bassiana* (Naturalis-L, Bio-Power)	L	30-day dietary and contact studies had no significant effect; LC_{50} (23 days, ingestion) 9.285 µg/bee	Copping (2004)
Apis mellifera	*B. brongniartii*	F	No negative effects noticed	Wallner (1988)
Arthropod and nematode populations	*B. bassiana* (Naturalis-L)	F	Chlorpyrifos had a stronger negative impact than the microbial treatment	Wang et al. (2001)
Bembidion lampros *Agonum dorsale*	*B. bassiana*	F/L	A negligible number was infected; low susceptibility of both species	Riedel and Steenberg (1998)
Bombus terrestris	*B. bassiana*	L/F	Able to infect bumblebees; it appears that there are no risks if the fungus is incorporated into soil or sprayed onto plants that are not attractive to bumblebees	Hokkanen et al. (2003)
Carabidae: *Calanthus micropterus* *C. piceus* *Carabus violaceus* *Cychrus caraboides* *Leistus ruefescens* *Nebria brevicollis,* *Pterostichus oblongopunctatus,* *P. niger*	*B. bassiana*	L	No adverse effects noticed	Hicks et al. (2001)
Carabidae, Staphylinidae	*B. bassiana*	F	Infection levels in adult ground beetles and rove beetles were low (Carabidae max. 7.6% and Staphylinidae max. 7.0%); an epizootic in the staphylinid *Anotylus rugosus* (67%) and *Gyrohypnus angustatus* (37%) was observed	Steenberg et al. (1995)

Table V (*Continued*)

Beneficial organism	Fungus (Strain/ Formulation)	Lab./ Field Trials (L/F)	Results/Observations	Reference
Cephalonomia tarsalis	*B. bassiana*	–	3 h exposure to 100 and 500 mg kg^{-1} wheat resulted in 52.5 and 68.6% mortality	Lord (2001)
Chrysoperla carnea	*B. bassiana*	L	Temperature, starvation and nutrition stresses significantly affected the susceptibility; nutrition stress caused the most increase in adult and larval mortality	Donegan and Lighthart (1989)
Coleomegilla maculate	*B. bassiana* (isolate ARSEF 3113)	L/F	No mortality was observed	Pingel and Lewis (1996)
Coleomegilla maculate and *Eriopis connexa*	*B. bassiana* (isolate ARSEF 731)	L	Mortality after direct application of spores; exposure via sprayed leaf surfaces resulted in no infection	Magalhaes et al. (1988)
Coleomegilla maculate lengi	*B. bassiana* (10 isolates)	L	6 isolates were highly virulent, 3 isolates caused low mortality	Todorova et al. (2000)
Diadegma semiclausum	*B. bassiana*	L	Detrimental effects on cocoon production and emergence depending on concentration	Furlong (2004)
Formica polyctena	*B. brongniartii*	F	No negative effects noticed	Dombrow (1988)
Earthworms: *Lumbricus terrestris* and others	*B. brongniartii* (commercial product of barley grains)	L/F	No effect in lab and in field noticed	Hozzank et al. (2003)
Earthworms: *Lumbricus terrestris*	*B. brongniartii*	L	No effect on earth worms noticed	Arregger-Zavadil (1992)
Earthworms: *Aporrectodea caliginosa*	*B. bassiana* (Bb64)	L	No effect on hatching rate of cocoons	Nuutinen et al. (1991)
Lysiphlebus testaceipe Aphidius colmani	*B. bassiana*	F	No significant impacts on both parasitoids	Murphy et al. (1999)
Megachile rotundata	*B. bassiana* (strain for grasshopper control)	L	Spray-application of flowering alfalfa in pots: female and male mortality averaged 9%; no difference in treatment and control; however *B. bassiana* grew out from dead bees	Goettel and Johnson (1992)
Nontarget arthropods (forests)	*B. brongniartii*	F	Only 1.1% of 10.165 collected insects and spiders were infected	Baltensweiler and Cerutti (1986)
Nontarget arthropods (forests)	*B. brongniartii*	F	1671 nontarget specimens were collected: 3.4% of them were infected, mainly species from Araneae, Thysanoptera, Homoptera, Coleoptera and Lepidoptera	Back et al. (1988)
Nontarget arthropods (major predators, parasitoids and pollinators on rangeland)	*B. bassiana* (strain GHA)	F	No statistical differences in the abundance of aerial insects	Brinkman and Fuller (1999)

Table V (*Continued*)

Beneficial organism	Fungus (Strain/ Formulation)	Lab./ Field Trials (L/F)	Results/Observations	Reference
Nontarget arthropods (forests)	*B. bassiana* (emulsifiable concentrate)	F	From 3615 invertebrates collected, only 2.8% became infected; *B. bassiana* could be applied to forest soil without a significant negative impact on forest-dwelling invertebrate population	Parker et al. (1997)
Non-target beetle communities	*B. bassiana* (strain SP 16)	F	No detectable effects	Ivie et al. (2002)
Perillus bioculatus	*B. bassiana* (six isolates)	L	5 isolates were highly pathogenic, isolate IPP46 showed low pathogenicity	Todorova et al. (2002)
Pimelia senegalensis *Trachyderma hispida* *Bracon hebetor* *Apoanagyrus lopezi*	*B. bassiana*	L	No infection in *P. senegalensis* and *T. hispida*; 100% mortality in the parasitoids *B. hebetor* and *A. lopezi*	Danfa et al. (1999)
Poecilus versicolor	*B. brongniartii* (Melocont-Pilzgerste, Melocont-WP, and Melocont-WG)	L	No significant negative effects on *P. versicolor* could be observed	Traugott et al. (2005)
Predatory mites: *O. insidiosus*	*B. bassiana* (Botanigard ES)	F	Can be used	Shipp et al. (2003)
A. colemani *Dacnusa sibiria* ***Parasites:*** *Encarsia formosa* *Eretmocerus eremicus* *Aphidoletes aphidimyza*			Not recommended during application of *B.bassiana* Used with caution during application of *B. bassiana*	
Prorops nasuta	*B. bassiana* (3 isolates)	L	Strain 25 caused the lowest infection level	De La Rosa et al. (2000)
Serangium parcesetosum	*B. bassiana*	L	The predator had significantly lower survivorship when sprayed with *B. bassiana* than with *P. fumosoroseus*; feeding on *B. bassiana* contaminated prey caused 86% mortality	Poprawski et al. (1998)

Beauveria species have been proven to be non-toxic and non-infectious to vertebrates, however, in a few cases, infections of *B. bassiana* have also been noticed.

Fish

Safety tests against fish for *B. bassiana* were reported for the isolate GHA by Goettel and Jaronski (1997) and for the product Naturalis-L® as well as for *B. brongniartii* by Copping (2004). No adverse effects of strain GHA were observed in embryos and larvae of the fish *Pimephales promelas*, when exposed for 31 days to 1×10^9 CFU L^{-1}. Naturalis-L® did not affect fish embryos, larvae or adults; the LC_{50} (31 days) for rainbow trout was 7300 mg L^{-1}. In contrast, when developing embryos of the inland

silverside fish, *Menidia beryllina*, were exposed to conidia of *B. bassiana*, various adverse effects were observed in embryos and larvae (Genthner & Middaugh 1992; Middaugh & Genthner 1994). In a strain of *B. brongniartii* (IMBST 95.031 and 95.041, Austria) the LC_{50} (at 30 days) for rainbow trout was 7200 mg L^{-1}, while the no observed effect level (NOEL) at 30 days was 3000 mg L^{-1} (Copping 2004).

Amphibia

A fungal suspension of *B. bassiana* was fed to the leopard frog, *Rana pipiens* via gastric incubation. The dosage was 9.8×10^8 conidia corresponding to 2.22×10^{12} conidia for a 70-kg human. No mortality or fungus recovery was recorded in any of the tissues. The viscera were free of mycelial growth. Viability of spores was established in fecal washings of pellets (Donovan-Peluso et al. 1980).

Reptiles

A fungus attributed to be *B. bassiana* was observed to cause infections in a captive American alligator (Fromtling et al. 1979), and *B. bassiana* was implicated in causing a pulmonary disease in captive tortoises (Georg et al. 1962; Gonzales-Cabo 1995). The reptiles were in captivity and under temperature stress which may explain their susceptibility to the fungus. When a tortoise was kept at 22°C and injected with 0.5 mL of 10^6 spores of *B. bassiana* into the lung, no mortality was observed, while a second contaminated tortoise died when kept only at 16°C (see Müller-Kögler 1967).

Birds

Birds may become exposed to entomopathogenic fungi directly by consuming spores deposited on their food, or indirectly by consuming fungus-infected insects. The concern about possible side-effects in birds is more than 100 years old. Müller-Kögler (1967) mentioned that according to E. Devaux (in Giard 1892), chickens, fed white grubs of *Melolontha* sp. infected with *B. brongniartii* (*B. tenella*), did not demonstrate any side-effects. However, precise examinations were not carried out at that time. Later, young *Falco sparvensis* were fed with 5×10^6 spores of *B. bassiana* per kg body weight (Althouse et al. 1997). No differences were found among any treatments and the control in growth, body mass or survival. Male and female ring-necked pheasants (*Phasianus colchicus*) were challenged per os with conidia of *B. bassiana* (Johnson et al. 2002). In both sexes, the weight gain at 17 and 25 days was not significantly different between challenged and control groups. Histopathological changes were generally undetectable. In 1987, a large field trial was carried out in Germany with *B. brongniartii* blastospores against the forest cockchafer *Melolontha hippocastani* ($1.5–2.8 \times 10^{14}$ blastospores ha^{-1}). During this experiment, no side-effects on birds, especially young ones, were noticed (Havelka & Ruge 1988). According to Copping (2004), the non-target bird toxicity for *B. bassiana* strain ATCC 74040 is: Oral LD_{50} (5 days) for quail >2000 mg kg^{-1} daily (by gavage); for *B. brongniartii* strain IMBST 95.031 and 95.041 (Austria): Dietary LD_{50} (5 days) for quail and mallard ducks >4000 mg kg^{-1}.

Effects on mammals and human health

Safety of entomopathogenic fungi, especially *B. bassiana* and *B. brongniartii*, to mammals and humans is of primary concern and has to be considered as one of the main potential hazards of using fungi as biocontrol agents. Therefore, it is not unusual that allergic, pathogenic or toxic risks for humans and mammals have been stressed in many papers (Steinhaus 1957; Müller-Kögler 1967; Ignoffo 1973; Austwick 1980; Burges 1981; Saik et al. 1990; Siegel & Shadduck 1990; Goettel et al. 1997, 2001; Vestergaard et al. 2003). Recently, some papers from South Korea on the addition of *B. bassiana* to human food documents a totally new aspect of this fungus. Yoon et al. (2003b) reported that extracts of *B. bassiana* synnemata had anticoagulant and immune system modulating activity, which could provide beneficial physiological activities for humans. In another paper, Yoon et al. (2003a) found that *B. bassiana* synnemata could be used as an additive to wheat flour for the preparation of noodle and bread.

Allergy

Allergies are caused by certain protein and polysaccharide antigens, so all types of microorganisms are potentially allergenic to man. Generally, a wide range of allergic reactions to various fungi can occur. According to Nolard (2004), 5–15% of the population suffering from respiratory allergy have been sensitised to one or several moulds. Exposure to fungus associated antigens may cause sensitisation, and later exposures can elicit reactions like respiratory distress, lachrymation or erythema. The main route of sensitisation is respiratory. Allergies relevant to the safety of biological pesticides are placed in Type I and III. Type I allergy is an immediate response to relatively small amounts of an inhaled allergen resulting in rhinitis, heavy asthma or lacrymation. Type III allergy is a delayed response 4–8 h after exposure to a relatively heavy inhaled dose of the allergen, causing fever, headache and weakness (Austwick 1980; Burges 1981). Basics of fungal allergy are summarised by Gumowski et al. (1991).

Nolard (2004) differenciates between 'outdoor air fungal allergy', 'indoor air fungal allergy' and 'fungal allergies in work environments'. Concerning *B. bassiana* and *B. brongniartii*, allergies or allergic reactions could occur in workers in production facilities who are exposed repeatedly to high concentrations of spores and when the fungi are released into the air in an inhalable form after application for biocontrol purposes. Because the conidia of both fungi are 'dry', relatively small (2–3 μm, globose, in *B. bassiana* and 2–6 μm, oval, in *B. brongniartii*) and produced in dusty clusters, they are easily spread by air and may reach the lower respiratory tract after inhalation (Austwick 1980).

The natural occurrence of *B. bassiana* in the air has already been mentioned in *Natural occurrence and geographical distribution* and *Mobility and persistence in air*. MacLeod (1954) reported, that *B. bassiana* was found within the lung tissues of 14 rodents. But, histological examination has not shown that the fungus could readily create a pathological condition within the tissues. In a survey of fungi from sputum of patients hospitalised from chronic pulmonary disease, more than 15% of more than 3000 sputum samples were found to contain one or more colonies of *B. bassiana* (Pore et al. 1970) and from 103 sputum specimens from male telephone workers, five contained *Beauveria* sp. (Comstock et al. 1974). Gumowski et al. (1991) listed about

100 fungal genera associated with allergy; *B. bassiana* was not mentioned at that time. However, there are some documents that indicate *B. bassiana* may cause allergies and/or allergic reactions. Müller-Kögler (1967) mentions several cases of allergic reactions in humans caused by this species, especially during the production process. Generally, headache, weakness and fever were noticed. High fever and a reaction similar to an anaphylactic shock were noticed by Dr Samšiňáková, Praha, and her assistant in 1965 when producing a spore powder of *B. bassiana*. However, when she was working with dry and dusty blastospores of *B. bassiana*, such reactions were never observed. These observations confirm that fungal conidia contain more allergenicity causing factors compared to hyphae and to submerged culture produced blastospores (Müller-Kögler 1967). Allergic reactions in workers were also reported by Mel'nikova and Murza (1980). In contrast to these findings, no incidents of human hypersensitivity reaction were noticed by workers of the company Mycotech during many years of mass production of the fungus (Goettel & Jaronski 1997). Inhalation experiments with *B. brongniartii* (*B. tenella*) and albino-mice ('Rüdiger') were reported by Müller-Kögler (1967). In preliminary experiments, mice were treated two times per week by dusting 50 mg conidia in a 10-L flask. They died within a short time. But the reactions were less harmful after only one treatment per week. There is no doubt, that the spore dose was rather high corresponding to 5 g m^{-3}. After histological examination, the fungus was not found in mouse lungs. In further inhalation experiments with *B. bassiana* and various animals, the fungus did not migrate from the lungs into other organs, and the lungs were completely rid of the fungus 4–5 days later (Mel'nikova & Murza 1980). Some inhalation and irritant data on *B. bassiana* and *B. brongniartii* are also presented by Copping (2004). *Beauveria* bassiana strain ATCC 74040: inhalation: LC_{50}: rats $>1.2 \times 10^8$ CFU/animal. Possible irritant to eyes, skin and respiratory system. *Beauveria brongniartii* strain IMBST 95.031 and 95.041 (Austria): Acute dermal LD_{50}: rats >2000 mg kg^{-1}. Mildly irritant to skin of rabbits.

These investigations and findings reveal that conidia of *Beauveria* species have allergenic potential. In molecular studies, *B. bassiana* crude extracts possess numerous IgE reactive proteins, some of which are cross-reactive among allergens from other fungi (Westwood et al. 2005). A strongly reactive potential *B. bassiana* specific allergen (35 kDa) was identified and confirmed by intradermal skin testing.

Pathogenicity/Toxicity

Besides allergy, one of the main concerns in the use of entomopathogenic fungi is the risk of infection to humans or mammals. On the other hand, *B. bassiana* itself or fungus diseased larvae of *Bombyx mori* have been used as medicants for hundreds of years in Chinese medicine (Müller-Kögler 1965).

Natural occurrence. Beauveria species have been rarely identified as agents of human infections, and in an overview on the emergence of less common, but medically important fungal pathogens on recipients, the genus *Beauveria* is not mentioned (Walsh et al. 2004; Strasser & Kirchmair 2006). Nevertheless, there are some cases where *B. bassiana* has been reported as the cause of mycotic keratitis in humans (Sachs et al. 1985; Low et al. 1997; Kisla et al. 2000; Sigler 2003) and in a rabbit cornea (Ishibashi et al. 1987). For example, Sachs et al. (1985) described the first case of *B. bassiana* keratitis in a patient following the removal of a corneal foreign body.

However, the patient was treated with topical corticosteroids and antibiotics prior to the identification of mycotic elements. This therapy may have depressed the normal protective mechanisms. A fungal keratitis due to *B. bassiana* was also described in an 82-year-old woman (Kisla et al. 2000). The patient was treated successfully with topical natamycin and oral fluconazole.

The first documented human deep tissue infection with a *Beauveria* sp. in a patient receiving immunosuppressive therapy was reported by Henke et al. (2002). Antifungal therapy with itraconazole (200 mg orally twice daily) was successful. Recently, a second case of disseminated *Beauveria* infection in an immunosuppressed patient with acute lymphoblastic leukemia was reported from New Zealand (Tucker et al. 2004). The infection was successfully treated with amphotericin B and itraconazole. In both cases, the *Beauveria* isolates were unable to grow at 37°C, and the New Zealand isolate even did not grow at 35°C. In both cases, the patients were not exposed directly to the fungus, e.g. by use of a *B. bassiana* product. Thus, we can only speculate how these patients came into contact with the fungus. Tucker et al. (2004) hypothesised that their patient was exposed to *B. bassiana* while living in an agricultural area. Recently, the first case of empyema caused by *B. bassiana* in a 51-year-old man was reported in Turkey (Gürcan et al. 2006). The authors think that prolonged air leakage after lung operation was the primary cause of infection. The patient recovered without any antifungal treatment after the air leakage was secured. The isolated strain of *B. bassiana* did not grow at 37°C.

Experimental data. During the development and registration of *B. bassiana* products for biocontrol, the fungus has been extensively tested for safety against several mammals. The first experiments were conducted by Schaerffenberg (1968). They included injection, inhalation and feeding tests with adult white rats. No toxic or pathogenic but allergic reactions were noticed. A commercially used strain of *B. bassiana* was administered to albino rats intragastrically and intraperitoneally and to rabbits intravenously (Mel'nikova & Murza 1980). The LD_{50} number of spores per animal was more than 1.1×10^{10}, 2.2×10^{10} and 4.0×10^{10}, respectively. Single administration of Boverin® dust intraperitoneally and intragastrically to albino rats resulted in an LD_{50} of 0.6 ± 0.1 g kg^{-1} and more than 10 g kg^{-1}, respectively. The potential pathogenicity of B. bassiana to mice was studied by intramuscular injection of 2×10^8 (high) and 2×10^5 (low) conidia (Semalulu et al. 1992). It was concluded that *B. bassiana* does not cause infection, multiply, nor survive for more than 3 days when injected into healthy mice.

Summaries and EPA evaluations of toxicity/pathogenicity data are available for *B. bassiana* strains GHA and ATCC 74040 on the EPA website: http://www.epa.gov/oppbppd1/biopesticides/ingredients/tech_docs/tech_128924.htm and /tech_128818.htm. The experiences of Mycotech Corporation (now Laverlam International, Butte MT) with the safety testing of their *B. bassiana* strain GHA are also presented by Goettel and Jaronski (1997). No infectivity or toxicity was demonstrated in any of the tests with rats and other vertebrates, The fungus was cleared from rats within 7 days after intratracheal application and within 3 days after intraperitoneal or peroral application. In an ocular irritation test with conidial powder, a moderate reaction, such as redness or swelling with no signs of infection was observed. Ocular introduction of two formulations, an oil flowable and an emulsifiable suspension,

resulted in minimal irritation and dermal application of conidia to rabbits showed only transitory erythema.

Some precise data on mammalian pathogenicity/toxicity of both *Beauveria* species are also presented by Copping (2004): *B. bassiana* strain ATCC 74040: Mammalian toxicity: No infectivity or pathogenicity was observed in rats after 21 days exposure to 1.8×10^9 colony forming units kg^{-1}. Acute oral LD_{50}: rats $>18 \times 10^8$ CFU kg^{-1}. Acute dermal LD_{50}: rats >2000 mg kg^{-1}. Inhalation: LC_{50}: rats $>1.2 \times 10^8$ CFU/animal. Skin and eye: Possible irritant to eyes, skin and respiratory system. Dermal, oral and inhalation studies with Naturalis-L® on rats indicated that the fungus is non-toxic and non-pathogenic. *Beauveria brongniartii* strain IMBST 95.031 and 95.041 (Austria): Acute oral LD_{50}: rats >5000 mg kg^{-1}. In rats, there was no toxicity, infectivity or pathogenicity from a single dose of 1.1×10^9 CFU kg^{-1}.

Conclusions

Beauveria bassiana and *B. brongniartii* (=*B. tenella*) are two well-known entomopathogenic fungi which have a worldwide distribution, and are used for biological control of pest insects for more than 100 years. This means that tons of fungus material of both species have been produced and used during that time. The products have passed the registration requirements in several countries, and are still widely used for biocontrol of pest insects. The present review documents, that there is a broad knowledge on *B. bassiana* and *B. brongniartii*, i.e. on their biology, their fate and behaviour in the environment, their effects on non-target organisms and on vertebrates, including mammals and humans. So far, no serious detrimental effects have been observed after application of these fungi. On the basis of our actual and presented knowledge, both *Beauveria* species, *B. bassiana* and *B. brongniartii*, should be considered as safe. Nevertheless, to avoid possible risks, certain vertebrate pathogenicity/toxicity tests and relevant studies on non-target organisms should be made within future registrations of new strains. Additionally, known protection measures during the production process and application are necessary to avoid allergic reactions. However, all risks can never be excluded. It is hoped, that this review provides a fundamental basis of knowledge for scientists, as well as for producers, potential registrants and regulatory authorities for their future work and decisions on the development and registration of these two fungi.

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Influence of Ultraviolet Light Protectants on Persistence of the Entomopathogenic Fungus, *Beauveria bassiana*

G. DOUGLAS INGLIS,*,†,[1] MARK S. GOETTEL,* AND DAN L. JOHNSON*

*Agriculture and Agri-Food Canada Research Centre, P.O. Box 3000, Lethbridge, Alberta, Canada T1J 4B1; and †Centre for Pest Management, Simon Fraser University, Burnaby, British Columbia, V5A 1S6

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The effect of ultraviolet light (uv) protectants on persistence of conidia of the entomopathogenic fungus *Beauveria bassiana* was investigated in laboratory and field environments. The survival of conidia applied in water onto glass coverslips or crested wheatgrass (*Agropyron cristatum*) leaves was reduced by greater than 95% after 15 min exposure to uv-B radiation in a controlled environment. Substitution of oil for water increased the survival of conidia on both substrates. However, conidial survival in oil was more pronounced on glass (74% mortality after 60 min) than on leaves (97% mortality after 60 min). The decreased protection provided by oil on leaves was attributed to spreading and/or absorption of the oil by the leaf tissues. None of 21 potential sunscreen formulations were toxic to nongerminated conidia *in vitro*. On wheatgrass leaves, 5 of the 12 water-compatible and two of the nine oil-compatible formulations enhanced survival of conidia after 3 h exposure to uv-B radiation in a controlled environment. Four water-compatible and three oil-compatible sunscreen adjuvants were subsequently tested in a repeated field experiment. The water-compatible fluorescent brightener, Tinopal LPW (conidial survival slopes of −2.1 and −1.7 in trials one and two, respectively), and a clay emulsion (slopes of −2.5 and −2.0) significantly increased survival of conidia compared to the water control (slopes of −3.3 and −2.7), whereas Congo Red (slopes of −3.1 and −2.8) and the optical brightener, Blankophor BSU (slopes of −4.2 and −3.7), were ineffective. Conidial survival in the field was not enhanced by the three oil-compatible adjuvants tested (oxybenzone, octyl-salicylate, and ethyl-cinnamate). The use of uv-B protectants in formulations can increase conidial survival and may enhance the efficacy of *B. bassiana* for controlling insect pests in epigeal habitats.

KEY WORDS: ***Beauveria bassiana;* persistence; ultraviolet radiation; uv-B; sunscreens; protectants; crested wheatgrass; *Agropyron cristatum;* formulation oil.**

INTRODUCTION

The entomopathogenic fungus *Beauveria bassiana* (Bals.) Vuill. has shown considerable potential for the management of insect pests (Feng *et al.*, 1994). Grasshoppers are economically important pests in arid agroecosystems, and recently the pathogenicity of *B. bassiana* has been demonstrated in a field environment against grasshoppers (Johnson and Goettel, 1993). However, *B. bassiana* conidia are hyaline and rapidly killed by sunlight (Daoust and Pereira, 1986; Inglis *et al.*, 1993). Since a threshold of inoculum is required to cause beauveriosis in insects, the inactivation of conidia by sunlight could seriously decrease the efficacy of conidia applied on foliage. If epigeal habitats are to be targeted for the management of insect populations with *B. bassiana,* methods to increase the persistence of conidia are needed.

The ultraviolet radiation-B (uv-B; 280-320 nm) component of sunlight is detrimental to all microorganisms (Tevini, 1993). A number of substances have been used to protect and enhance the persistence of entomopathogenic viruses (Ignoffo and Batzer, 1971; Shapiro *et al.*, 1983; Martignoni and Iwai, 1985; Shapiro, 1989, 1992), *Bacillus thuringiensis* Berliner (Morris, 1983; Cohen *et al.*, 1991), the entomopathogenic fungus *Metarhizium flavoviride* Gams & Rozsypal (Moore *et al.*, 1993), and the nematode *Steinernema carpocapsae* (Weiser) (Nickle and Shapiro, 1992) exposed to artificial uv-B radiation. However, the efficacy of uv-B protectants for increasing the persistence of fungal propagules in a field environment has not been previously studied. Therefore, the objectives of this study were to: (1) test and compare water and paraffinic oil formulations for uv protection of *B. bassiana* conidia; (2) screen a number of potential uv protectants in the laboratory; and (3) test their efficacy in a field environment.

[1] To whom correspondence should be addressed at Agriculture and Agri-Food Canada, Research Centre, P.O. Box 3000, Lethbridge, Alberta, Canada T1J 4B1. Fax: (403) 382-3156.

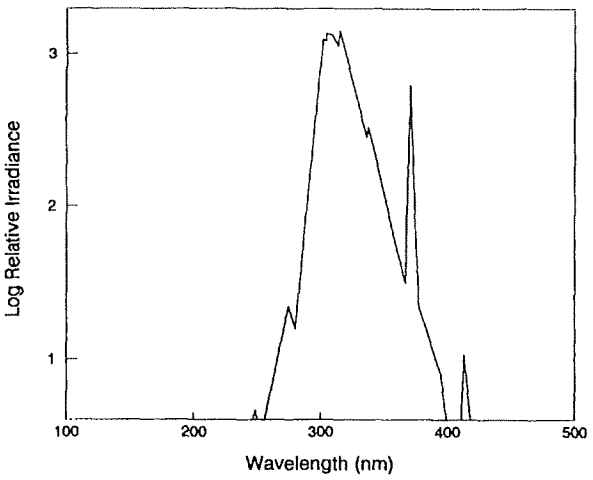

FIG. 1. Spectral distribution of irradiative energy from the Ultra-Lum uv-B fluorescent bulb used for irradiating *Beauveria bassiana* conidia.

MATERIALS AND METHODS

Conidial Inoculum

Immediately before use, dry conidia of *B. bassiana* (GHA strain, supplied by Mycotech Corp., Butte, MT) were suspended in sterile deionized water or in paraffinic formulation oil using a Kontes mechanical pestle or a Potter–Elvehjem homogenizer. In the laboratory experiments, a paraffinic emulsifiable oil was used, whereas a paraffinic oil flowable (no emulsifier added) was used in the field experiment. Conidial concentrations were estimated with a hemocytometer and adjusted as required.

Influence of Substrate

The survival of conidia exposed to uv-B radiation on leaves and on glass was compared. Conidia (2×10^5 conidia/μl) suspended in water or in oil were pipetted (1 μl) onto sterile round coverslips (13 mm in diameter) and onto the surface of leaf pieces (approximately 2×0.5 cm) of field-collected crested wheatgrass (*Agropyron cristatum* L.) attached to a white plastic tray using double-sided tape. The coverslips and leaf pieces were then placed 10 cm below a uv-B fluorescent bulb (Ultra-Lum, Carson, CA) for 0, 15, 30, 45, or 60 min at 25 ± 1°C. Radiation from this bulb ranges in wavelength from 260 to 400 nm, with a peak near 300 to 320 nm (Fig. 1). Intensity of uv-B radiation was measured using a UVX radiometer (UVP Inc., San Gabriel, CA) equipped with a UVX-31 sensor (310 nm peak); uv flux at a distance of 10 cm ranged from 601 to 675 μW/cm^2 along the length of the bulb. Following exposure, leaf pieces and coverslips were individually placed in 5 ml of 0.01 *M* phosphate buffer with 0.05% Tween 80 (pH, 7.0) in 20-ml scintillation vials and washed at ambient temperature for 2 h on a rotary shaker at 300 rpm. The wash solution was then diluted in a 10-fold dilution series and 100-μl aliquots for each dilution were spread on a semiselective oatmeal–dodine agar medium (consisting of 17.5 g oatmeal agar, 2.5 g agar, 0.45 g Cyprex (dodine), 2.5 mg crystal violet, 0.2 g penicillin, and 0.5 g streptomycin in 500 ml of deionized water) (Chase *et al.*, 1986). Cultures were incubated at 25°C for 6 to 7 days and the number of colony-forming units (CFU) were enumerated at the dilution yielding 30 to 300 CFU per dish. Conidial survival relative to the control (time$_0$) was calculated as (CFU T_0 − CFU T_x/CFU T_0) × 100. An initial experiment indicated that incubation of conidia in water and oil at 25°C in the dark for up to 6 h had no effect on conidial viability.

Sunscreens

The adjuvants tested were divided into water- or oil-compatible compounds. Water-compatible adjuvants consisted of eight stilbene brighteners provided by Dr. M. Shapiro, USDA-ARS, Beltsville, Maryland; Congo Red (Sigma Chemical Co., St. Louis, MO); and diethanolamine-4-methoxycinnamate (Nipasorb D; Graessorb D; Nipa Laboratories, Clwyd, UK). In addition, an attapulgite clay formulation (consisting of 15% oil (v/v), 12% clay (w/v) and 73% deionized water) and a 5% oil–water emulsion formulation (v/v) were tested. The oil-compatible adjuvants used were: butyl-methoxy-dibenzoylmethane (Parsol 1789; Givaudan & Co. Ltd., Surrey, UK); ethyl trans-cinnamate (Sigma); 4-isopropyl-dibenzoylmethane (Eusolex 8020; Merck, Darmstadt, Germany); magnesium silicate (Florisil 60-100 mesh; Fisher Scientific, Edmonton, AB, Canada); octyl-*p*-methoxycinnamate (Parsol MCX; Givaudan); octyl-salicylate (Graessorb S; Nipa Laboratories); 2-hydroxy-4-methoxybenzophenone (oxybenzone; Sigma); and 2,2-dihydroxy-4-methoxybenzophenone and 2,2-hydroxy-4-octoxybenzophenone supplied by Mycotech.

Solubility and Toxicity of Sunscreens

The solubilities of the sunscreens were determined in water and oil at room temperature (20 to 22°C). The maximum sunscreen concentration tested was 5%; saturated solutions at room temperature were used if sunscreens were insoluble at 5%. To measure possible toxicity, nongerminated conidia (2×10^5 conidia/μl) were suspended in each of the sunscreens. After 12 h at 25°C, 1-μl aliquots were pipetted onto sterile coverslips, and the coverslips were washed and CFU enumerated on oatmeal–dodine agar as previously described.

Sunscreen Selection

Conidia were suspended in each of the formulations so that the final concentration was 2×10^5 conidia/

μl; sunscreen concentrations were the same as for the toxicity experiment. Within 15 min, conidial suspensions (1 μl) were pipetted onto the surfaces of leaf pieces taped to a plastic tray and the leaf pieces were exposed to uv-B radiation for 1.5 and 3.0 h. The leaf pieces were washed and viable conidia were enumerated on oatmeal–dodine agar as previously described. Control treatments consisted of conidia applied in oil or water onto leaf pieces that were either exposed to uv-B radiation or maintained in the dark for the same period.

Field Evaluations

Conidia in various uv protectants were sprayed on crested wheatgrass in a field at the Agriculture and Agri-Food Canada Research Centre, Lethbridge. The field had been seeded in 1989 at a rate of 11.1 kg/ha with rows 17.5 cm apart and mowed in April 1992, 3 months prior to commencement of the experiment. Treatments were arranged in a randomized complete block design with three replicate plots measuring 3.0 by 1.5 m. The target concentration was 3.0×10^{13} conidia/ha or 1.4×10^{10} conidia/plot. The water treatments were Tinopal LPW (Calcofluor white; M_2R; 5% w/v), Blankophor BSU (5% w/v), Congo Red (5% w/v), clay (12% w/v), and water alone. Tinopal LPW was obtained by adjusting the pH of Blankophor, BBH, to 9.5 with 1 *N* potassium hydroxide. The oil treatments consisted of oxybenzone (5% w/v), ethyl-cinnamate (5% v/v), octyl-salicylate (5% v/v) and oil alone. An uninoculated control treatment was also included.

Water-compatible formulations were applied at a rate of 100 litres/ha (45 ml/plot), using a compressed CO_2 (40 PSI) bicycle sprayer (R&D Sprayers Inc., Opelousas, LA) equipped with three 015-F80 nozzles (Lurmark Ltd., Longstanton, Cambridge, UK). To obtain the optimal spray pattern, the height of the boom was adjusted according to the height of the wheatgrass canopy. Oil-compatible formulations were applied at a rate of 5 litres/ha (2.25 ml/plot) with an ultralow volume (ULV) spinning disk sprayer (Micron Sprayers Ltd., Bromyard, UK) operated at 7000 rpm. To eliminate drift, plots were enclosed in a polyethylene tent ($3.0 \times 1.5 \times 1.5$ m) during the ULV spray application. Water- and oil-sensitive papers were randomly placed on the soil surface in selected plots to evaluate the spray distribution. Conidia were applied at times of low wind velocity (<4 m/s) on the morning of July 28, 1993 for trial one and August 12 for trial two.

Ten leaves from the top of the canopy were randomly collected from the centre of each of the three replicate plots. Times of sampling were immediately after (time 0), and 1, 2, 4, 6, 8, 12, and 16 days postapplication. Care was taken to choose older leaves that would have been present at the time of conidial application for the 6- to 16-day sample times. Within 1 h of collection, leaves were transported to the laboratory in plastic bags and each of the 10 leaves per replicate were aseptically cut across the laminae into pieces of about 1 cm long. Propagules were recovered from the leaf pieces using the wash, dilution-spread-plate technique on oatmeal–dodine agar as described previously. Following the washing the total area of the leaf pieces were determined with a leaf area meter (Model 3100, Li-Cor Inc., Lincoln, NE) and the mean number of CFU/cm^2 of leaf area was calculated. Sample leaf areas per plot averaged 6.07 and 6.72 cm^2 in trials one and two, respectively. To confirm the identity of *B. bassiana,* representative colonies were isolated, grown in slide culture, and examined microscopically.

Weather Data

Mean hourly incoming solar radiation (300 to 2800 nm), temperature, relative humidity, precipitation, and wind direction and velocity were recorded at a weather station adjacent to the field plots. The pyranometer malfunctioned 4 and 5 days after application of conidia in trial one. Hours of bright sunshine during these two days were 13.4 and 8.7 h, respectively (Environment Canada, Lethbridge). Using hours of bright sunshine, theoretical total incoming solar radiation at ground level (Q_s theoretical) on these days was calculated using the equations of Baier and Robertson (1965) and Robertson (1968) as 3.17×10^4 kJ/m^2 for Day 4 and 2.40×10^4 kJ/m^2 for Day 5. These values were higher than those recorded by the pyranometer (Q_s actual) for days with comparable hours of bright sunshine. Therefore, Q_s theoretical was plotted against Q_s actual for the period of July 28 to August 28, 1993. The coefficient of determination (r^2) observed was 0.96 and the equation used to describe this relationship was Q_s actual = 3967.9 + 1.08(Q_s theoretical); standard errors of the mean (SE) were 903.4 and 0.044 kJ/m^2 for a and b, respectively. From the equation, Q_s was estimated as 2.58×10^4 kJ/m^2 for Day 4 and 1.87×10^4 kJ/m^2 for Day 5.

Statistical Analyses

All computations were performed using the GLM, REG, and TTEST procedures (SAS Institute, 1988). Residuals were plotted against predicted values and where necessary the appropriate transformations were used to normalize the data. The conidial population data were always $\log_{10}$ transformed, and $\log_{10}$-values for the means and SE are presented throughout the text. In all instances, SE were calculated from individual treatments and are presented in parentheses. In two cases in the substrate selection experiment, the percentage reduction data (Table 1) were arcsine-transformed, but untransformed means and SE are presented in Table 1.

All experiments in controlled environments were arranged as completely randomized designs. The substrate selection experiment was analyzed as a split plot in time with two levels of formulation and substrate and five levels of time. This experiment was conducted three times for the water formulation and twice for the oil formulation; data from the trials were combined for analysis. The toxicity and selection experiments were repeated once, and with the exception of the clay formulation treatment, they were analyzed using one-way ANOVA. In conjunction with a significant F test, Tukey's studentized range test ($\alpha = 0.05$) was used to separate means. For the clay formulation, comparisons were made using the TTEST procedure.

The field experiment was arranged as a randomized complete block design. Water- and oil-compatible formulations were analyzed separately as a split plot in time. When a significant ($P \leq 0.05$) interaction was observed for formulation and time, pairwise comparisons of the slopes of conidial persistence between the control and test formulation were conducted using analysis of covariance with $\log_{10}$-transformed time used as the covariate. For the SAS REG procedure, the mean persistence data for each formulation were used to fit linear models; $\log_{10}$-transformation of the x-axis was used for water-compatible formulations in both trials and for the oil-compatible formulations in trial one but not in trial two. In addition to time, the predictability of cumulative solar radiation on conidial survival was examined by regression analysis. In most instances, no *B. bassiana* conidia were recovered from leaf segments collected from untreated plots, and when conidia were recovered, it was at very low levels (<10 CFU/cm^2). Therefore, the uninoculated control treatment was excluded from the analyses of conidial persistence.

RESULTS

Influence of Substrate

On both leaves and coverslips, droplets of water were localized and evaporated within 15 min of placement. Oil droplets (4 to 5 mm in diameter) covered a larger area than did water droplets (1.5 to 2.0 mm in diameter) on coverslips. On wheatgrass leaves, oil spread rapidly across the lamina and an oil sheen was usually observed.

When exposed to uv-B radiation in the laboratory, significant interactions were observed between formulation (oil and water) and duration of exposure ($F = 8.2$; $df = 4,112$; $P \leq 0.0001$), and formulation and substrate ($F = 13.0$; $df = 1,19$; $P = 0.0019$). Comparisons between the oil and water formulations for individual substrates indicated that conidial survival was enhanced in oil on glass (Table 1); formulation ($F = 65.4$; $df = 1,5$; $P = 0.0005$), time ($F = 23.4$; $df = 4,56$; $P \leq 0.0001$), and the interaction between formulation and time ($F = 8.5$; $df = 4,56$; $P \leq 0.0001$) were significant. Although there was no interaction ($F = 1.95$; $df = 4,56$; $P = 0.11$) between formulation and time, more conidia ($F = 8.3$; $df = 1,5$; $P = 0.035$) were recovered from leaves treated with conidia in oil (averaged over time) than with conidia in water.

Comparisons between substrates for individual formulations indicated that the survival of conidia applied in oil on coverslips was greater ($F = 21.5$; $df = 1,5$; $P = 0.0056$) than the survival of conidia applied in oil to leaves (Table 1). Time alone was highly significant ($F = 8.6$; $df = 4,40$; $P \leq 0.0001$), and there was no interaction ($F = 1.3$; $df = 4,40$; $P = 0.29$) between time and substrate. In water, there was no difference ($F = 0.74$; $df = 1,9$; $P = 0.41$) in survival of conidia applied to either substrate (Table 1). As with oil, there was no interaction ($F = 2.0$; $df = 4,72$; $P = 0.10$) between time and substrate and time alone was highly significant ($F = 46.6$; $df = 4,72$; $P \leq 0.0001$).

Solubility and Toxicity of Sunscreens

Of the adjuvants tested, 5 of 10 and 6 of 9 were highly soluble in water and oil (>5% w/v), respectively. The Blankophor brighteners, BBH, DML, HRS, LPG, and RKH were marginally soluble in water, and Florisil, HOB, and HMB exhibited low solubility in oil. Saturated formulations (room temperature) of the marginally soluble adjuvants were subsequently tested for uv protection. None of the water- ($F = 0.85$; $df = 10,11$; $P = 0.60$) or oil-compatible ($F = 1.19$; $df = 7,8$; $P = 0.40$) adjuvants tested were toxic to nongerminated conidia of *B. bassiana* after 12 h incubation at 25°C. The increased pH of Blankophor, BBH (9.5), required to enhance its solubility in water, had no effect on conidial viability.

Sunscreen Selection

After 1.5 h exposure to uv-B radiation, conidial survival in 9 of 11 water-compatible formulations was greater ($\alpha = 0.05$) than that of conidia exposed to uv-B radiation in water alone (Table 2). Conidial survival was equal to that of nonexposed conidia applied in water in all but three of the formulations. Five of the adjuvants protected conidia ($\alpha = 0.05$), exposed to uv-B radiation for 3 h. These included Congo Red and the optical brighteners, BSU, BBH, P167, and Tinopal LPW; BBH was tested at a concentration of only 0.25% (w/v). The clay formulation treatment was analyzed separately. There was no difference in survival ($T = 1.51$; $df = 10$; $P = 0.16$) between conidia exposed to uv-B for 1.5 h (3.74, SE = 0.20 log CFU/leaf) and conidia maintained in the dark (4.14, SE = 0.17 log CFU/leaf). In contrast, conidial survival in clay was reduced ($T = 2.57$; $df = 10$; $P = 0.028$) after 3.0 h exposure to uv-B.

TABLE 1

Influence of Formulation and Substrate on Survival of *Beauveria bassiana* Conidia Exposed to uv-B Radiation

Duration of exposure (min)	Glass (SE)		Leaves (SE)	
	$\log_{10}$ cfu[a]	% Reduction[b]	$\log_{10}$ CFU[a]	% Reduction[b]
Water formulation				
0	3.96 (0.06) a[c]	—	4.11 (0.06) a	—
15	2.46 (0.11) b	96.0 (0.78) a[d]	2.36 (0.28) b	96.1 (1.0) a[d]
30	1.59 (0.27) bc	99.0 (0.27) b	1.70 (0.30) bc	98.8 (0.42) ab
45	0.66 (0.34) c	99.3 (0.41) b	1.66 (0.31) bc	98.6 (0.66) b
60	1.12 (0.31) c	99.4 (0.26) b	0.74 (0.30) c	99.7 (0.10) b
Oil formulation				
0	3.99 (0.08) a	—	3.75 (0.15) a	—
15	3.92 (0.07) ab	22.4 (6.0) a	3.24 (0.22) a	49.2 (18.0) a
30	3.58 (0.20) ab	49.9 (16.4) ab	2.64 (0.54) ab	75.7 (7.1) ab
45	3.32 (0.27) ab	62.2 (14.5) ab	2.80 (0.24) ab	82.2 (5.2) ab
60	3.13 (0.26) b	74.4 (9.3) b	1.79 (0.38) b	97.4 (0.89) b

[a] Conidia ($\log_{10}$ colony-forming units (CFU)) recovered from glass coverslips or crested wheatgrass leaves. Values in parentheses following means represent standard errors of the means.

[b] Percentage reduction was calculated as $((CFU_{T0} - CFU_{Tx})/CFU_{T0})100$.

[c] Means not followed by the same letter within each formulation-substrate group are significantly different ($\alpha = 0.05$) according to Tukey's studentized range test. The experiment was conducted three times for the water formulation ($n = 10$) and two times for the oil formulation ($n = 6$).

[d] Data were arcsine transformed.

From leaves treated with conidia in clay and exposed to uv-B for 3.0 h, 4.19 (0.11) log CFU/leaf were recovered compared to 4.55 (0.089) log CFU/leaf from nonexposed leaves. Congo Red, clay, Tinopal LPW, and Blankophor, BSU, were selected for evaluation in the field experiment.

None of the nine oil-compatible formulations tested enhanced ($\alpha = 0.05$) survival relative to oil alone after 1.5 h exposure to uv-B radiation (Table 3). After 3.0 h exposure to uv-B radiation, conidial survival in Parsol MCX and 2,2-hydroxy-4-octoxybenzophenone was superior ($\alpha = 0.05$) to the survival of conidia applied in

TABLE 2

Influence of Water-Compatible Formulation Adjuvants on Survival of *Beauveria bassiana* Conidia Applied to Leaves and Exposed to uv-B Radiation for 1.5 and 3.0 h

Formulation	Concentration (%)	$\log_{10}$ CFU/leaf[a] (SE)	
		1.5 h	3.0 h
BSU-Optical brightener (OB)[b]	5	4.68 (0.04) a[c]	4.41 (0.07) a[c]
Congo Red[b]	5	4.58 (0.03) a	4.45 (0.10) a
Tinopal LPW-OB[b]	5	4.46 (0.06) a	4.12 (0.12) ab
BBH-OB	0.25	4.35 (0.15) a	4.22 (0.12) ab
P167-OB	5	4.27 (0.31) a	4.43 (0.04) a
HRS-OB	2	4.13 (0.10) ab	3.49 (0.15) abc
LPG-OB	0.25	4.01 (0.14) ab	3.55 (0.23) abc
DML-OB	0.25	4.10 (0.05) ab	3.18 (0.32) bc
RKH-OB	0.25	2.86 (0.34) b	3.27 (0.32) abc
Diethanolamine-4-methoxycinnamate	5	1.16 (0.52) c	0.41 (0.41) d
Oil emulsion	5	0.74 (0.49) c	0.53 (0.53) d
Water	—	1.10 (0.52) c	2.77 (0.16) c
Water (no uv Exposure)	—	4.68 (0.06) a	4.44 (0.06) a

[a] Conidia ($\log_{10}$ CFU/leaf) recovered from crested wheatgrass leaves.

[b] Adjuvants selected for subsequent field evaluations.

[c] Means not followed by the same letter are significantly different ($\alpha = 0.05$), according to Tukey's studentized range test. The experiment was conducted two times ($n = 6$).

TABLE 3

Influence of Oil-Compatible Formulation Adjuvants on Survival of *Beauveria bassiana* Conidia Applied to Leaves and Exposed to uv-B Radiation for 1.5 and 3.0 h

Formulation	Concentration (%)	$\log_{10}$ CFU/leaf[a] (SE) 1.5 h	3.0 h
Oxybenzone[b]	5	3.71 (0.17) ab[c]	1.40 (0.47) bcd[c]
Ethyl-cinnamate[b]	5	3.44 (0.17) ab	1.19 (0.38) bcd
2,2-Dihydroxy-4-methoxybenzophenone	2	3.36 (0.22) ab	1.75 (0.35) abc
Parsol MCX	5	3.21 (0.23) ab	2.49 (0.50) ab
Octyl-salicylate[b]	5	3.16 (0.41) ab	0.85 (0.35) bcd
Eusolex	5	2.96 (0.35) ab	1.64 (0.37) abcd
Parsol 1789	5	2.82 (0.04) ab	0.00 (0.00) d
2,2-Hydroxy-4-octoxybenzophenone	4	2.31 (0.77) b	2.37 (0.13) ab
Florisil	2	2.17 (0.48) b	1.19 (0.38) bcd
Oil	—	2.51 (0.17) b	0.50 (0.50) cd
Oil (No uv exposure)	—	4.41 (0.18) a	3.36 (0.14) a

[a] Conidia ($\log_{10}$ CFU/leaf) recovered from crested wheatgrass leaves.
[b] Adjuvants selected for subsequent field evaluations.
[c] Means not followed by the same letter are significantly different ($\alpha = 0.05$), according to Tukey's studentized range test. The experiment was conducted two times ($n = 6$).

oil alone. On the basis of availability and a previous report of their efficacy in protecting *Metarhizium flavoviride* conidia from artificial uv-B radiation (Moore *et al.*, 1993), oxybenzone, ethyl-cinnamate, and octyl-salicylate were selected for evaluation in the field experiment.

Field Evaluations

Conditions of incoming solar radiation, temperature, precipitation, and relative humidity fluctuated within and between trials (Fig. 2). Total incoming solar radiation was 3.72×10^5 kJ/m^2 in trial one and 25.1% less (2.79×10^5 kJ/m^2) in trial two. Hourly incoming solar radiation, averaged over the 16 days of the trials (daylight hours), was 1430 (74.9) and 1327 (62.6) kJ/m^2, respectively. Mean hourly temperatures were 16.2 (0.29) and 15.2 (0.23) °C, and relative humidities averaged 67.8 (1.1) and 75.8 (0.94) % in trials one and two, respectively. Five periods of light precipitation (<3.0 mm per event) were recorded in trial one. In trial two, 51 mm of rain fell 3 to 5 days after application, followed by two additional periods of light rain (<1 mm).

Of the five water-compatible formulations, there was no difference ($F = 1.37$ and 1.60; $df = 4,8$; $P = 0.33$ and $P = 0.26$) in conidial populations among the formulations immediately after application (T_0) in either trial one or two; populations ranged from 4.10 (0.12) to 5.05 (0.21) log CFU/cm^2. For all the water-compatible formulations, conidial survival declined logarithmically over time (Figs. 3 and 4), and the persistence data were fitted to linear regressions following log-transformation of both the CFU and the time data. Coefficients of

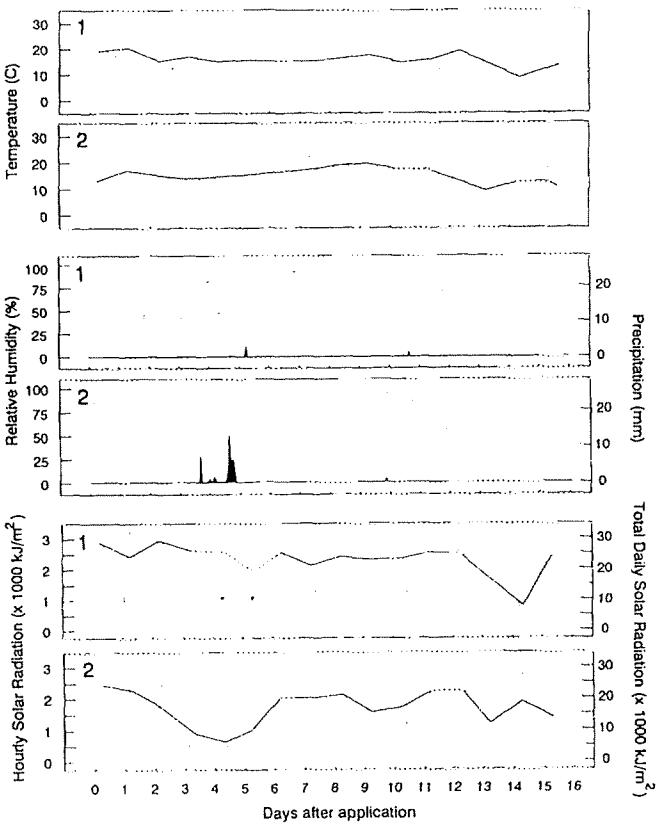

FIG. 2. Hourly (dotted lines) and mean (solid lines) temperature (°C), relative humidity (%), precipitation (peaks), hourly (dotted lines) and total daily (solid lines) solar radiation (kJ/m^2; 300–2800 nm) during trials one and two. Asterisks represent missing solar radiation data; daily solar radiation during this period (dashed line) was estimated using hours of bright sunshine.

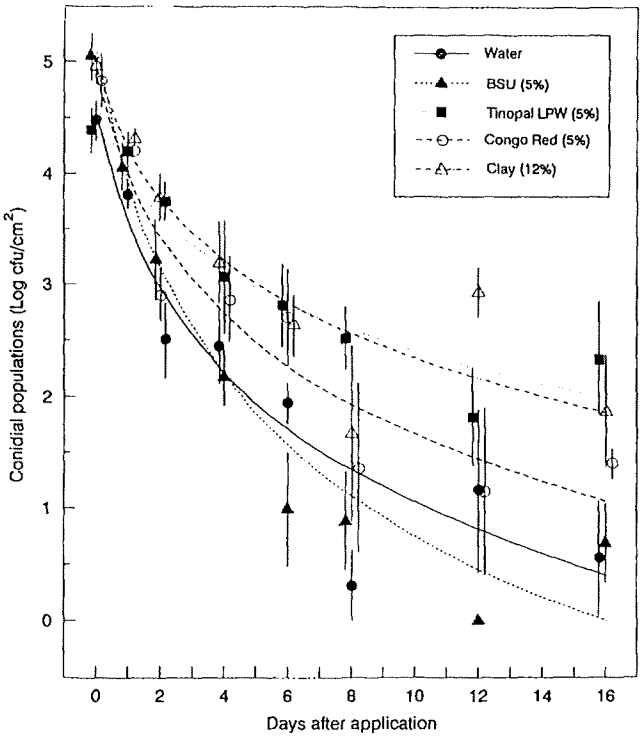

FIG. 3. Persistence of *Beauveria bassiana* conidia in water-compatible sunscreens on crested wheatgrass in trial one (July 28 to August 13, 1993). Populations were quantified as $\log_{10}$ colony-forming units (CFU)/cm^2 of leaf area and vertical lines represent standard errors of means (n = 3). To avoid superimposition of standard error bars, means are offset along the x-axis.

determination (r^2) ranged from 0.84 to 0.95 and from 0.85 to 0.98 for trials one and two, respectively (Table 4). Slopes of conidial persistence for the formulations ranged from −1.7 to −4.2, and both slopes (T = −5.6 to −16.9; df = 6; $P \leq 0.0013$) and y-intercepts (T = 12.0 to 36.5; df = 6; $P \leq 0.0001$) were significantly different from zero. For individual treatments, neither slopes nor y-intercepts differed (F = 0.24 to 2.96; df = 1,44; $P \geq 0.09$) between trials. Although a strong relationship was observed between cumulative solar radiation and conidial persistence, light was generally a less effective predictor of conidial survival (r^2 = 0.67 to 0.98) than was time (log-transformed). However, in trial two for the clay formulation, a stronger relationship was observed between conidial persistence and light (r^2 = 0.98) than with time (r^2 = 0.85).

In both trials, time (F = 67.3 and F = 75.8; df = 7,70; $P \leq 0.0001$) and formulation (F = 9.97 and F = 20.6; df = 4,8; P = 0.0034 and P = 0.0003) were significant, as were the interactions (F = 1.84 and F = 1.91; df = 28,70; P = 0.021 and P = 0.013) between them. Pairwise comparisons of the formulations with the control treatment using analysis of covariance indicated that Tinopal LPW (F = 6.19 and F = 8.42; df = 1,44; P = 0.0167 and P = 0.0058) and clay (F = 4.30 and F = 3.97; df = 1,44; P = 0.0439 and P = 0.0527) enhanced survival of conidia in both trials (Figs. 3 and 4) and that neither formulation was superior (F = 0.60 and F = 0.62; df = 1,44; P = 0.44 and P = 0.43). Congo Red did not affect (F = 0.21 and F = 0.04; df = 1,44; P = 0.65 and P = 0.85) the persistence of conidia in either trial. Although similar (F = 2.88; df = 1,44; P = 0.097) to the control treatment in trial one, the survival of conidia in BSU was less (F = 7.74; df = 1,44; P = 0.0079) than in water in trial two.

Immediately after application, conidial populations were similar (F = 0.10 and F = 0.29; df = 3,6; P = 0.96 and P = 0.82) in the four oil-compatible formulations in both trials. In contrast to the water-compatible formulations, slopes of conidial persistence differed between the trials. In trial one, best fit regression analysis required a $\log_{10}$-transformation of both CFU and time data, and slopes of conidial persistence ranged from −3.1 to −3.8 (Table 4). In contrast, only the CFU and not the time data required log-transformation in trial two; slopes ranged from −0.20 to −0.23. In both trials, all slopes (T = −6.4 to −24.8; df = 6; $P \leq 0.0003$) and y-intercepts (T = 14.7 to 62.1; df = 6; $P \leq 0.0001$)

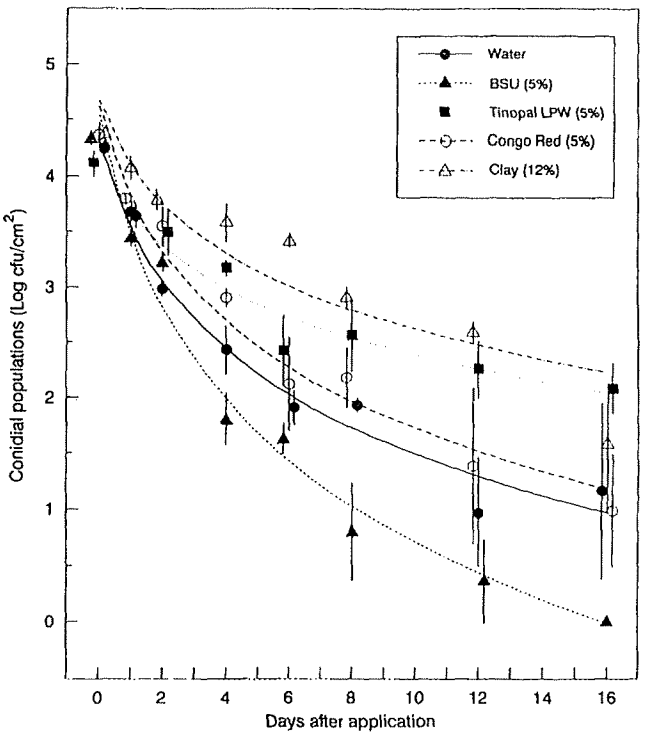

FIG. 4. Persistence of *Beauveria bassiana* conidia in water-compatible sunscreens on crested wheatgrass in trial two (August 12 to 28). Populations were quantified as $\log_{10}$ colony-forming units (CFU)/cm^2 of leaf area and vertical lines represent standard errors of means (n = 3). To avoid superimposition of standard error bars, means are offset along the x-axis.

TABLE 4

Linear Regression Data for *Beauveria bassiana* Conidial Persistence on Crested Wheatgrass Leaves in the Field Experiment[a]

	Trial one					Trial two				
	a	SE(a)	b	SE(b)	r^2	a	SE(a)	b	SE(b)	r^2
Water-compatible										
Water	4.50[b]	0.37	−3.33[b]	0.46	0.90	4.31	0.14	−2.71	0.17	0.98
BSU	5.08	0.32	−4.19	0.40	0.95	4.53	0.18	−3.68	0.22	0.98
Tinopal LPW	4.59	0.19	−2.11	0.24	0.93	4.18	0.12	−1.73	0.14	0.96
Congo Red	4.85	0.30	−3.08	0.37	0.92	4.62	0.16	−2.78	0.20	0.97
Clay	4.92	0.36	−2.49	0.44	0.84	4.66	0.27	−1.96	0.33	0.86
Oil-compatible										
Oil	5.03	0.31	−3.83	0.39	0.94	4.18	0.16	−0.20	0.02	0.94
Oxybenzone	5.08	0.21	−3.07	0.26	0.96	4.49	0.12	−0.23	0.02	0.97
Ethyl-cinnamate	5.06	0.18	−3.43	0.22	0.98	4.54	0.07	−0.23	0.01	0.99
Octyl-salicylate	4.89	0.33	−3.06	0.41	0.90	4.41	0.09	−0.22	0.01	0.98

[a] For the water-compatible formulations in both trials and the oil-compatible formulations in trial one, the x-axis was $\log_{10}$-transformed and the regression equation is: $\log_{10}$ CFU/cm^2 = $a + b$ ($\log_{10}$ days + 1), where $n = 8$. For the oil-compatible formulations in trial two, the x-axis was untransformed and the regression equation used is: $\log_{10}$ CFU/cm^2 = $a + b$ (days).

[b] All y-intercepts and slopes are significantly different from zero ($P \leq 0.01$).

were significantly different from zero. Coefficients of determination for the oil-compatible formulations ranged from 0.90 to 0.99 (Table 4). As with the water-formulations, a strong relationship was observed between cumulative light and conidial persistence for oil ($r^2 = 0.85$ and 0.94), oxybenzone ($r^2 = 0.95$ and 0.97), ethyl-cinnamate ($r^2 = 0.95$ and 0.99), and octyl-salicylate ($r^2 = 0.74$ and 0.98). For the oil and octyl-salicylate formulations in trial one, and all formulations in trial two, light was almost as good a predictor of conidial survival as was time.

For the oil-compatible formulations, time ($F = 51.0$ and $F = 87.8$; $df = 7,56$; $P \leq 0.0001$) but not formulation ($F = 3.65$ and $F = 0.80$; $df = 3,6$; $P = 0.083$ and $P = 0.53$) influenced conidial persistence, and there was no interaction ($F = 0.59$ and $F = 0.44$; $df = 21,56$; $P = 0.91$ and $P = 0.98$) between formulation and time (Figs. 5 and 6). Comparison of water and oil controls over time indicated no difference in conidial persistence in trial one ($F = 0.48$; $df = 7,28$; $P = 0.84$) or two ($F = 1.1$; $df = 7,28$; $P = 0.36$).

DISCUSSION

The impact of uv-B radiation on fungal populations in natural habitats has not been extensively studied. *B. bassiana,* a hyaline fungus, is soil-borne and is rarely isolated from plant foliage. Furthermore, the survival of *B. bassiana* conidia applied on foliage is very poor (Inglis *et al.,* 1993), and the most important parameter limiting survival of conidia in epigeal habitats appears to be sunlight (Daoust and Pereira, 1986; Inglis *et al.,* 1993). Ultraviolet radiation causes primary (i.e., nucleic acid mutations) and/or secondary (i.e., photoreactions) damage to exposed microorganisms, either of which may lead to cellular death (Tevini, 1993). We observed that conidia of *B. bassiana* are highly sensitive to artificial uv-B radiation but the mechanism causing death is unknown. It seems likely that the extreme sensitivity of *B. bassiana* to the uv-B portion of the solar spectrum limits its persistence in epigeal habitats. Formulation of *B. bassiana* to provide protection from ultraviolet light will be necessary to increase its survival and efficacy in epigeal habitats.

We found that Congo Red, clay, and all of the stilbene brighteners tested provided a degree of protection from artificial uv-B radiation (Table 2). Four of the adjuvants were tested in a field environment but only clay and the stilbene brightener, Tinopal LPW, consistently increased the persistence of *B. bassiana* conidia on wheatgrass leaves. Congo Red and the stilbene brightener, BSU, were ineffective. Stilbene brighteners readily absorb uv-B radiation and have previously been shown to protect nuclear polyhedrosis virus (NPV) occlusion bodies (Martignoni and Iwai, 1985; Shapiro, 1992), *Steinernema carpocapsae* (Nickle and Shapiro, 1992), and *B. thuringiensis* (Morris, 1983) from uv-B inactivation. Tinopal LPW has also been shown to enhance the virulence of NPV (Shapiro and Dougherty, 1993). In contrast to the uv-B absorbing adjuvants, clay acts as a sunlight blocker. Sunlight blockers have provided effective protection of viruses from uv-B radiation (Ignoffo and Batzer, 1971; Jaques, 1971; Shapiro *et al.,* 1983), and the use of blockers such as clay, starch, or carbon may be preferable because they are either environmentally innocuous or easily decomposed.

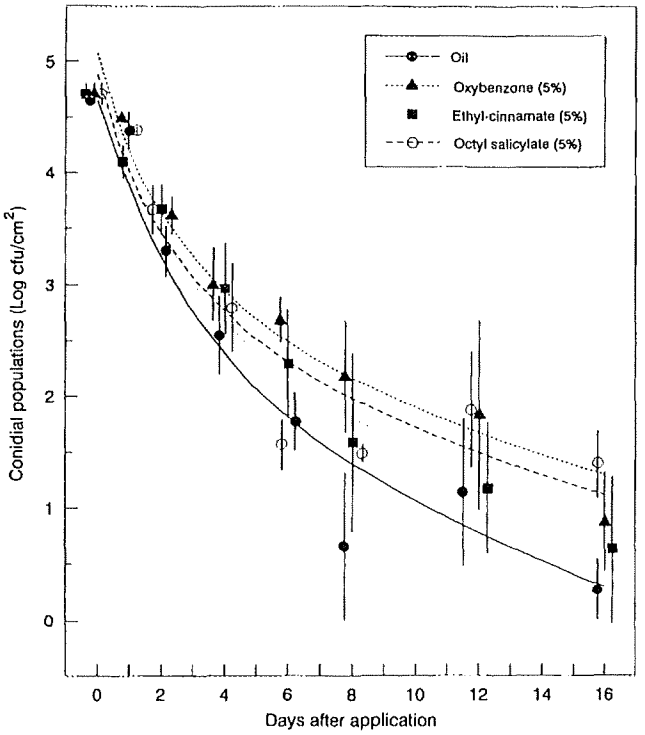

FIG. 5. Persistence of *Beauveria bassiana* conidia in oil-compatible sunscreens on crested wheatgrass in trial one (July 28 to August 13, 1993). Populations were quantified as $\log_{10}$ colony-forming units (CFU)/cm^2 of leaf area and vertical lines represent standard errors of means (n = 3). To avoid superimposition of standard error bars, means are offset along the x-axis.

Several entomopathogens, including *B. bassiana,* have been applied in oil at ultralow volumes in attempts to increase their efficacy (Feng *et al.,* 1994). Similarly to a previous field study (Inglis *et al.,* 1993), we found no marked increase in the survival of *B. bassiana* conidia applied in oil at ULV relative to conidia sprayed in water at conventional volumes. However, in our laboratory experiment, we observed that *B. bassiana* conidia exposed to uv-B radiation survived better in oil than in water on glass. Conidia of *M. flavoviride* in oil on glass were also found to be less sensitive to uv radiation (below 305 nm) than were conidia in water; this was attributed to absorption of uv radiation by the oil (Moore *et al.,* 1993). Although we observed that survival of conidia was also greater on leaves in oil than in water, conidial persistence in oil was substantially reduced on leaves relative to that on glass. Despite the presence of an oil sheen, we attributed the decreased efficacy of oil on leaves, at least in part, to the absorption of oil into mesophyll cells. Under field conditions, the volume of oil deposited on leaves at ULV is considerably less than the volume of oil (1 μl) that we pipetted onto the leaf segments in the laboratory. Therefore, it would be expected that the rate and degree of absorption of oil into leaf tissues applied at ULV would be greater, and the rapid absorption of oil into leaf tissues may explain the poor protection of *B. bassiana* conidia from uv-B radiation that we observed on leaves in the field experiment.

Oil-soluble sunscreens, which absorb uv-B radiation, have been developed for use in the cosmetic industry (Shaath, 1990). Several of these (oxybenzone, ethyl-cinnamate, and octyl-salicylate) were found to protect *M. flavoviride* conidia on glass (Moore *et al.,* 1993). Although several of the oil-compatible adjuvants we tested protected *B. bassiana* conidia from artificial uv-B radiation, they did not protect conidia under field conditions. Reasons for the differential efficacy of these adjuvants between the field and the laboratory are unknown. However, absorption of the oil carrier into the leaf tissues may have contributed to the decreased protection provided by these adjuvants in the field.

This study demonstrated that *B. bassiana* conidia are extremely sensitive to uv-B radiation and that the survival of conidia can be prolonged in field environments by using uv-B protectants. The results also confirm that solar radiation, and in particular the uv-B portion of the solar spectrum, is important in limiting

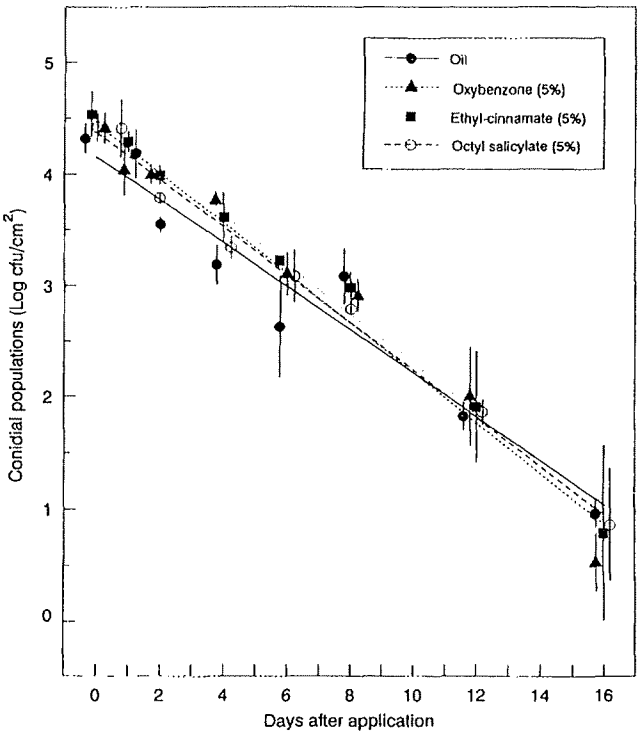

FIG. 6. Persistence of *Beauveria bassiana* conidia in oil-compatible sunscreens on crested wheatgrass phylloplanes in trial two (August 12 to 28). Populations were quantified as $\log_{10}$ colony-forming units (CFU)/cm^2 of leaf area and vertical lines represent standard errors of means (n = 3). To avoid superimposition of standard error bars, means are offset along the x-axis.

conidial survival in epigeal habitats. However, the utilization of uv-B protectants to increase the efficacy of insect control will depend on whether sunscreens prolong the survival of conidia sufficiently to enhance efficacy. Future research should focus on efficacy tests against insects and on the identification of more effective uv-B protectants and/or formulation strategies.

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ORIGINAL ARTICLE

THE INFLUENCE OF ULTRAVIOLET LIGHT ON PATHOGENICITY OF ENTOMOPATHOGENIC FUNGUS *BEAUVERIA BASSIANA* (BALSAMO) VUILLEMIN TO THE EUROPEAN CORN BORER, *OSTRINIA NUBILALIS* HBN. (LEPIDOPTERA: CRAMBIDAE)

CAGÁŇ[1]*, Ľ., M. ŠVERCEL[2]

ABSTRACT

The influence of different doses of ultraviolet (UV) light on the pathogenicity of entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin to the European corn borer, *Ostrinia nubilalis* Hbn., and radial growth of fungus was studied in laboratory conditions. The suspensions of *B. bassiana* isolate SK99 were exposed to UV light. Four different doses of UV light were used in the experiment. The distance between exposed suspensions and UV light source was 0.3 m. Exposure duration was 15, 30, 45 and 60 minutes (as A, B, C and D variants). Control variant SK99 and obtained variants SK99A, SK99B, SK99C and SK99D were cultivated 21 days on Sabourard-dextrose agar. The larvae of *O. nubilalis* were infected with dry powder consisted of mycelia and spores from fungus cultures. During 10 days, the mortality of infected larvae was evaluated. It was ascertained that UV light exposition significantly influenced the mortality effect of *B. bassiana* isolates to *O. nubilalis* larvae. Variant SK99C showed the highest level of infectivity. Radial growth of UV variants was slower with rising time of exposure. The best ability to grow possessed non-irradiated isolate SK99 and the worse variant SK99D. The difference between these two variants was significant.

KEY WORDS: Beauveria bassiana, UV light, mutagenesis, Ostrinia nubilalis

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PATHOGENICITY OF ENTOMOPATHOGENIC FUNGUS BEAUVERIA BASSIANA (BALSAMO) VUILLEMIN

DETAILED ABSTRACT

V laboratórnych podmienkach sa sledoval vplyv rôznych dávok ultrafialového (UV) žiarenia na radiálny rast a patogenicitu entomopatogénnej huby *Beauveria bassiana* (Balsamo) Vuillemin vo vzťahu k vijačke kukuričnej, *Ostrinia nubilalis* Hbn.. Suspenzie izolátu *B. bassiana* označeného SK99 boli vystavené UV žiareniu. Zdrojom UV žiarenia bola baktericídna výbojka Philips TUV 30 W s hladinou radiácie 83 $\mu W.s/cm^2$ a vlnovou dĺžkou 253,7 nm. V pokuse sa použili štyri rôzne dávky UV žiarenia. Vzdialenosť medzi suspenziami a zdrojom UV žiarenia bola 0,3 m. Dĺžka expozície bola 15, 30, 45 a 60 minút (varianty A, B, C a D). Kontrolný variant SK99 a získané varianty SK99A, SK99B, SK99C a SK99D sa kultivovali 21 dní on Sabourard-dextrózovom agare. Larvy *O. nubilalis* boli infikované práškom zloženým z mycélia a spór z kultúr entomopatogénnej huby. Počas 10 dní sa hodnotila mortalita infikovaných lariev.

Všetky varianty si zachovali virulenciu proti larvám škodcu. Desať dní po infekcii lariev bola minimálna úmrtnosť (varianty SK99A a SK99B) 86,67%. Potvrdilo sa, že vystavenie UV žiareniu preukazne ovplyvnilo účinnosť *B. bassiana* proti larvám *O. nubilalis*. Variant SK99C mal najväčšiu infekčnú schopnosť. Po desiatich dňoch od jeho aplikácie dosiahla mortalita lariev 100%. Infekčná schopnosť spór z variantu SK99C bola preukazne vyššia ako v prípade variantov SK99A a SK99B. Medzi kontrolou (SK99) a variantom SK99C bol rozdiel v infektivite nepreukazný. S predlžujúcim sa časom expozície bol radiálny rast UV variantov bol pomalší. Najlepšiu schopnosť rastu mal pôvodný variant nevystavený UV žiareniu, najhoršiu variant vystavený UV žiareniu najdlhšie t. j. SK99D. Rozdiel medzi týmito dvomi variantami bol preukazný.

Všeobecne sa mutagenéza pomocou UV žiarenia často používa na produkciu mutantov húb. Výsledky práce potvrdili, že je reálne používať UV žiarenie na tvorbu nových kmeňov entomopatogénnej huby *B. bassiana.* Experiment s larvami sa nerobil hneď po aplikácii žiarenia, ale až o 21 dní. Predpokladá sa, že aj keď mohla prebehnúť autoreparácia poškodenej genetickej informácie, 21 dní je dosť dlhá doba na stabilizáciu genetických zmien.

INTRODUCTION

The selection of entomopathogenic microorganism is an important link in the use of effective biopreparations for the protection of plants from insect pests of agricultural crops [29].

The entomopathogenic filamentous fungus *B. bassiana* attacks many species of insects [11,15], including the European corn borer, *Ostrinia nubilalis* Hbn. Many authors provided experiments in an interaction between *O. nubilalis* population and *B. bassiana* in the field and laboratory conditions. They found out that the fungus is very significant agent to suppress survival of pest's grubs [1, 2, 3, 6, 10, 18, 23].

The influence and interactions between UV radiation and properties of fungus *B. bassiana* were evaluated in a lot of works. Tobar et al. [27] selected *B. bassiana* isolates for resistance to UV light. Their Isolate Bb9218 was resistant to 10, 30 and 60 min exposures to UV light and it showed the highest *Hypothenemus hampei* percentage mortality throughout the evaluation time and was significantly different to the other treatments. Teng [26] ascertained that a long time exposure very significantly suppressed fructification of fungus, but on the other side different strains and isolates had different level of sporulation in addiction on different time of UV light exposure. Conidia survival of *B. bassiana* is a function of time exposure characterised by S-curve. Exposure of the fungus to UV light can change the form of its nutrition from prototrophy to auxotrophy.

Level of production of proteolytic, lipolytic and chitinolytic enzymes as well as organic acids recorded in exposed cultures was different from a level of non-irradiated ones caused different level of production of metabolites like proteolytic, lipolytic and chitinolytic enzymes and organic acids [7].

Volume of reversion mutant escalated with rising level of UV light exposure [17]. Kirsanova and Usenko [16] tested the infectivity of *B. bassiana* to drozophila (*Drosophila melanogaster)* and elicited that the influence of UV irradiation is asymmetrical.

UV light exposure to *B. bassiana* cultures can interfere with their physiological properties.

Hegedus and Khachatourians [12, 14] clarified relationship between UV radiation and influence of temperature on morphological, physiological and biochemical properties of the fungus. They affirmed non-linear dependence between pathogenecity of fungus and doses of UV exposure.

There are known positive results in the breeding of *Beauveria* species with the application of mutagens. Tests showed that laser treatment of *Beauveria* spores increased the mortality rate of pine moths (*Dendrolimus* sp.). Stable strains of *Beauveria* were selected which were more toxic to pine moths, grew faster and produced more spores, with a greater resistance to UV light [28]. Two strains of *B. bassiana*, isolated from *Carposina sasakii* and the scarabaeid *Holotrichia parallela*, were selected for their substantial spore production. Irradiation produced 3 mutant strains with increased spore production and increased infectivity towards *C. sasakii.* These new characters were retained in mass production of the mutants and field test results are described as satisfactory [30].

Because black-pigmented conidia were more tolerant to stimulated sunlight, it was recommended that it may be possible to incorporate, by selection or genetic engineering, this phenotypic character into potential mycopesticides [14].

The aim of this work was to determine the influence of different doses of UV light to the pathogenicity of *B. bassiana* to *O. nubilalis* larvae and its radial growth on Sabouraud-Dextrose agar.

MATERIAL AND METHODS

B. bassiana, isolate SK99, (Department of Plant Protection, Slovak Agricultural University in Nitra), and larvae of *O. nubilalis* originated from Slovakian population bred in laboratory for more than three generations on semi-artificial diet [22] were used in the experiment.

The fungus was grown on Sabouraud-Dextrose agar (SDA) at 25ºC for 21 days. Conidia of the 21-day culture of the fungus served as the material for irradiation. The suspension of conidia filtered through cotton and sterile gauze in ceramic filter was used in the experiment. Portions of 20 ml of an aqueous suspension containing 10^6 per ml conidia were placed in Petri dishes and irradiated. The source of ultra-violet light rays was a bactericidal lamp Philips TUV 30 W with a dose rate at the level of irradiation 83 $\mu W.s/cm^2$ and wavelength of 253.7

nm. The distance between exposed suspensions and UV source was 0.3 m. Exposure duration was 15, 30, 45 and 60 minutes (as A, B, C and D variants). The irradiated (marked as SK99A, SK99B, SK99C, SK99D) and non-irradiated (SK99) suspensions were inoculated on SDA in Petri dishes and incubated at 25ºC for 21-days.

Petri dishes with incubated *B. bassiana* suspensions were used for pathogenicity tests and for radial growth analysis.

Each irradiated *B. bassiana* suspension was tested in 3 replications (Petri dishes) for its patogenicity against *O. nubilalis* larvae. Each Petri dish contained 10 fifth-instar larvae. Control variant was organised in the same way. The larvae were put into the Petri dishes containing the fungus for 5 minutes. Then they were removed in dishes with segmented maize leaves and maintained at 25°C. The mortality of larvae was recorded at 24 hours intervals during ten days. Dead larvae were put into Petri dish on the moistured filter paper to confirm the growth of *B. bassiana*. Analysis of variance was used for statistic evaluation of the experiment.

Disks 5 mm in diameter were taken from incubated suspensions for radial growth analysis of *B. bassiana* variants (SK99A-SK99D) and control variant (SK99).
They were put on SDA in Petri dishes and cultivated 6 days at 25°C. Each experiment was performed in 3 repetitions.

Following equation was used for calculation of daily radial growth of fungus cultures:

$$G = G_a - G_b,$$

G = radial growth,
a = time of measurement
b = a – 24

Analysis of variance was used for used for statistic evaluation of the experiment.

RESULTS

Insect test

Table 1 shows the mortality of the European corn borer larvae caused by UV light variants of *B. bassiana*. All of the variants tested were virulenced against the pest larvae. Ten days after larvae had been treated by *B. bassiana*, the minimum of their mortality was 86.67 % by variants SK99A and SK99B. The 45 minutes exposure by UV light positively influenced the infectivity of *B. bassiana* (variant SK99C). Variant SK99C had the highest infectivity after 10 days and mortality of tested larvae was 100.0 %. The analysis of variance revealed significant differences between variants SK99A, SK99B and SK99C after 10 days. The relation between level of mortality and doses of exposure was non-linear.

Table 1. Infectivity of ultra-violet light variants of *Beauveria bassiana* (SK99A, B, C, D) toward *Ostrinia nubilalis* larvae. Exposure of ultra-violet light duration was 15, 30, 45 and 60 minutes (as A, B, C and D variants). For details see material and methods. Values are given at the average of three repetitions, each with 10 larvae. Means marked with the same letter are not significantly different (P = 0.05, Tuckey´s multiple range test).

Variant	% mortality of larvae									
	1.day	2.day	3.day	4.day	5.day	6.day	7.day	8.day	9.day	10.day
SK99	0	3.33	13.33	23.33	30.00	56.67	60.00	63.33	73.33	93.33 bc
SK99a	0	6.67	16.67	30.00	40.00	63.33	73.33	80.00	80.00	86.67 b
SK99b	0	10.00	13.33	20.00	36.67	43.33	43.33	56.67	63.33	86.67 b
SK99c	0	16.67	23.33	33.33	40.00	76.67	80.00	80.00	96.67	100.00 c
SK99d	0	3.33	13.33	20.00	36.67	60.00	63.33	76.67	86.67	93.33 bc
Control	0	0	0	0	0	0	0	0	0	0 a

Radial growth

Exposure of the isolate SK 99 to different UV light doses caused decreased fungus ability to growth (Table 2). The growth all of UV light variants lag behind control variant SK 99. The level of fungus growth was continually depleted with the escalated doses of exposure. The highest significant differences were between non-exposed SK 99 and exposed SK 99 B and SK 99 D.

The differences in the growth rate are illustrated in Fig. 2. The fastest growth was achieved by non-irradiated variant SK 99 and, similarly like radial growth ability, was decreased with increased UV doses.

Table 2. Growth of *Beauveria bassiana* variants on Sabouraud-Dextrose agar in mm. Average – average daily accessories of mycelium. Exposure of ultra-violet light duration was 15, 30, 45 and 60 minutes (as A, B, C and D variants). For details see material and methods. Means marked with the same letter are not significantly different (p = 0.05 and p= 0.01; Tuckey´s multiple range test).

Variant	Average	95 % (p= 0.05)	99 % (p=0.01)
SK 99	1.84	A	A
SK 99 A	1.74	B	A B
SK 99 B	1.69	B C	B
SK 99 C	1.65	C	B C
SK 99 D	1.55	D	C

DISCUSSION

It is interesting that increasing doses of UV light decreased the infectivity of fungus by the finite level (A, B - level). But the fungus gained the highest level of patogenicity at C – exposure (45 minutes). When the exposure duration increased to 60 minutes, the mortality of grubs fallen down.

Kirsanova and Usenko [16] discovered that with rising dose of UV-rays, the yield of variants of the fungus increased with reduced virulence, but the relation between fungus infectivity and UV irradiation was non-symmetrical. The curve was fluctuated, similar like in our case. However, the test insect was drozophila (*Drosophila melanogaster).*

But, different strains and isolates of *B. bassiana* can not have the same response and susceptibility to UV light influence [26].

Müller-Kögler [21] discovered that conidia of fungus were not damaged after long UV exposure (after 60 minutes). Conversely, longer exposures (1.5 hour) conducted to faster growth and better spore production of fungus. Fructification of fungus decreased after 4.5 hours of UV light irradiation and after 30-48 hours was completely stopped.

According to Levitin et al. [17], the dependence of the survival of *B. bassiana´*s conidia to UV rays is characterised by a sigmoid curve. The survival of conidia decreases with increased doses of UV lights. With the frequency of mutations it is similar, but exist threshold here. Amount of mutants fell down after exceeding this threshold. We discovered that the increasing time of exposure caused reduced radial growth and growth rate of variants. That is in agreement with conidia survival described above.

In the experiment of Farques et al. [9], conidia from 65 isolates of *B. bassiana*, 23 of *Metarhizium anisopliae*, 14 of *Metarhizium flavoviride* and 33 isolates of *Paecilomyces fumosoroseus* were irradiated by artificial sunlight (295 to 1,100 nm at an ultraviolet-B irradiance of 0.3 Wm^2) for 0, 1, 2, 4 and 8 hours. Exposure for 2 h or more was detrimental to all isolates tested. Their results are the most similar to ours. In our preliminary trial it was necessary to find correct exposure time. It is a reason why we used in our experiment not longer than 60 minutes exposure.

It is difficult to compare the results concerned to correct time of UV light necessary for induction of mutations. Sharma et al [24] found that when *B. bassiana* was exposed to UV 4 hours, toxin production was higher probably due to some cell mutation. Morley et al. [20] used conidia of 14 isolates of the entomopathogenic fungi *B. bassiana*, *M. flavoviride* and *M. anisopliae* which were exposed to 4, 8, 16 and 24 hours of UV light from a sunlight simulator at 40 degree. Conidial viability decreased markedly in all isolates with increasing UV exposure. Germination ranged between 10 and 50% after 24 hours exposure to UV.

Generally, fungal mutants are often generated by UV-mutagenesis [4, 5, 19, 25]. Our results show that it is real possibility to use UV irradiation for development of new *B. bassiana* strains. We did not treat growth experiments next to irradiation, but after

21 days. It could also come to autoreparations of damaged genetic information, but 21 days seems to be enough long time for stabilisation of genetic changes in developed UV variants.

The reduction of growth ability of fungus could be caused by many reasons. We tested the growth ability, but other authors attained the change manner in nutrition, from prototrophy to auxotrophy [17]. It was recorded production of new metabolites like enzymes, toxins, pigments etc. or their reduction [7, 12, 13, 17]. They also determined differences in quality and quantity of fungus metabolites. However no relation between germination rates, radial growth, conidial production, medial lethal time and other colony characteristics of *B. bassiana* strains and their virulence was found during the study in Argentine [8].

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[1] Ľudovit Cagáň, ludovit.cagan@uniag.sk, * to whom correspondence should be adressed
[2] M. Švercel,
Department of Plant Protection, The Slovak Agricultural University in Nitra,
A. Hlinku 2, 94976 Nitra, Slovakia,
tel.: +421 37 65 08 253

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論　文（Original article）

Density dynamics of an entomopathogenic fungus, *Beauveria bassiana* introduced into fresh water

WANG Bin[2] and SHIMAZU Mitsuaki [1)*]

Abstract

Density dynamics of *Beauveria bassiana* in fresh water were investigated to detect possible influences of this fungus on aquatic ecosystems. Conidia of *B. bassiana* were inoculated in non-sterilized lake water, sterilized lake water, non-sterilized distilled water, and sterilized distilled water in the laboratory, and their densities monitored. The conidia decreased sharply in all experimental waters over a short time. More than 90% of the live conidia were lost within 28 d, only 0.03% survived in the non-sterilized lake water. No germinating conidium was found in non-sterilized waters after 27 d, while they had been observed in sterilized waters up to 84 d after inoculation. Other microorganisms existing in natural waters might inhibit germination of this fungus and reduce the densities of live conidia. The result testified the difficulty of *B. bassiana* accidentally contaminating natural fresh waters.

Key words : density, entomopathogenic fungus, aquatic ecosystem, germination, conidia

Introduction

Beauveria bassiana (Balsamo) Vuillemin has been regarded as an efficient biocontrol agent against many aggressive insect pests including immigrant disaster pests, and we have been studying this fungus as a control agent of the Japanese pine sawyer, *Monochamus alternatus* which vectors the pinewood nematode, pathogen of pine wilt disease (Shimazu et al., 1995; Shimazu et al., 2002a; b; Shimazu and Sato, 2003; Shimazu 2004a; b). To utilize this fungus as a microbial insecticide, dynamics of the fungus after application should be considered since it could persist in certain niches for a relatively long time. Shimazu (2002a) and Wang et al. (2002) studied the density dynamics of *B. bassiana* in forests. *Beauveria bassiana* conidia applied in the environment may accidentally enter aquatic ecosystems. Investigating the proliferation of *B. bassiana* in water will produce a good understanding of its life cycle and its possible influence on aquatic lives when the fungus accidentally enters fresh water. Up to now, the density dynamics of *B. bassiana* in water have not been understood. Therefore, we experimentally inoculated *B. bassiana* conidia in fresh water, and the density dynamics of the fungus were investigated.

Materials and Methods

Fungal isolate

A strain of *B. bassiana* F-263 isolated from a larva of the Japanese pine sawyer, *Monochamus alternatus* collected in Kumamoto prefecture of Japan in 1980, was used for the experiment. The isolate shows a high virulence to *M. alternatus* (Shimazu and Kushida, 1983) and has been studied for its use for the control of pine wilt disease by killing this insect (Okitsu et al., 2005; Shimazu et al., 1992, 1995). The isolate has been preserved in the Forest and Forestry Product Research Institute, Japan.

Experimental water and inoculation

Lake water and distilled water were used in the present study to assess proliferation ability of *B. bassiana* in fresh water. The lake water was collected from Lake Kasumigaura, the second largest lake in Japan on 17 May 2005 and kept at room temperature.

On the next day, collected lake water was roughly filtered with filter paper (Advantec #2), and then its natural pH was measured with an electronic pH meter. A portion of the experimental water was sterilized using a 0.2 μm cellulose nitrate filter (Sartorius AG) in order to assess the influence of microbes or other organisms on dynamics of *B. bassiana* conidia. Altogether four kinds of experimental waters were thus prepared; sterilized distilled water, non-sterilized distilled water, sterilized lake water and non-sterilized lake water (natural lake water). Since the natural pH of the lake water was 8.0, the pH of the distilled water for the experiment was also adjusted to 8.0 with Na_2CO_3 solution.

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* Department of Forest Entomology, Forestry and Forest Products Research Institute (FFPRI), 1 Matsunosato, Tsukuba, Ibaraki 305-8687 Japan; e-mail: shimazu@ffpri.affrc.go.jp
1)Department of Forest Entomology, Forestry and Forest Products Research Institute (FFPRI)
2) Present address: Provincial Key Laboratory of Microbial Control, Anhui Agricultural University, Hefei, Anhui, P. R. China

Preparation and inoculation of *B. bassiana* F-263

Beauveria bassiana F-263 was cultured on Sabouraud's dextrose agar with 1% yeast extract (abbreviation SDAY), at 25 ˚C for 3 weeks. The conidia were scraped from the culture with a sterile glass rod and suspended in sterilized water to obtain a conidial suspension. The suspension was filtered through a piece of tissue paper to remove conidial masses and mycelial fragments. The original concentration of the suspension was calculated using a hemocytometer.

This original high-density conidial suspension was added to each kind of experimental water at a final concentration at 1×10^6 conidia/ml. Two hundred milliliters of each experimental water containing conidia was poured into a 500 ml sterilized flask and capped with a Silicosen® culture plug (Shin-Etsu Polymer Co., Ltd.). Three replicates were prepared for each kind of water. All the flasks were statically incubated at 25˚C with an L/D cycle of 16/8.

Sampling

A small portion (1 ml) of each experimental water was sampled weekly, and diluted with sterile water to make a final concentration of approximately 1×10^3 conidia/ml. Onto a D0C2 selective medium plate (3 g Bactopeptone, 0.2 g $CuCl_2 \cdot {}_2H_2O$, 2 mg crystal violet, 15 g agar, 1000 ml distilled water, pH 10.0 with Na_2CO_3, developed by Shimazu and Sato, 1996), 0.1 ml of the diluted sample suspension was dropped and spread out with a sterilized glass rod. Three plates were prepared for one sample; altogether 9 plates were used for each experimental water sample. All the plates were incubated at 25˚C with continuous light for 6 d. The colony forming units (CFUs) on D0C2 plates were counted, and the colonies were identified. For identification of the colonies, 10 colonies were randomly transferred onto SDAY when more than 10 colonies appeared on a plate, and if less than 10, all of them were transferred, cultured and identified. The ratio of *B. bassiana* versus other species from SDAY cultures was used to estimate the true number of *B. bassiana* colonies. The day before sampling and culturing of the experimental waters, each water sample was also checked using a Thoma's hemocytometer to count the number of conidia and hyphae to investigate the rate of germinating conidia and to estimate the proper dilution ratio for culturing of the sampled water on the following day.

Results

Density dynamics of *B. bassiana*

All fungal cells of *B. bassiana* in four kinds of waters decreased sharply over 4 weeks (Fig. 1). Since the initial conidial densities of *B. bassiana* in four groups of water were not equal (one-way ANOVA, p=0.012), densities at each observation were compared with the initial densities of each flask. Decline of *B. bassiana* densities in the first week in non-sterilized lake water (72.62%) and non-sterilized distilled water (79.20%) were higher than those in sterilized lake water (52.65%) and in sterilized distilled water (25.01%). After 28d of survival, densities of *B. bassiana* in sterilized lake water, non-sterilized lake water, sterilized distilled water, and non-sterilized distilled water decreased by 91.92%, 99.97%, 93.94%, and 96.99%, respectively. The decline ratios of all treatments were over 90%, and the conidia in non-sterilized lake water lost their viability most rapidly. There was a significant difference in decline tendencies of the fungal density between sterilized and non-sterilized distilled waters (two-way Repeated-Measures ANOVA, p=0.0075 for waters and p<0.0001 for days × waters),

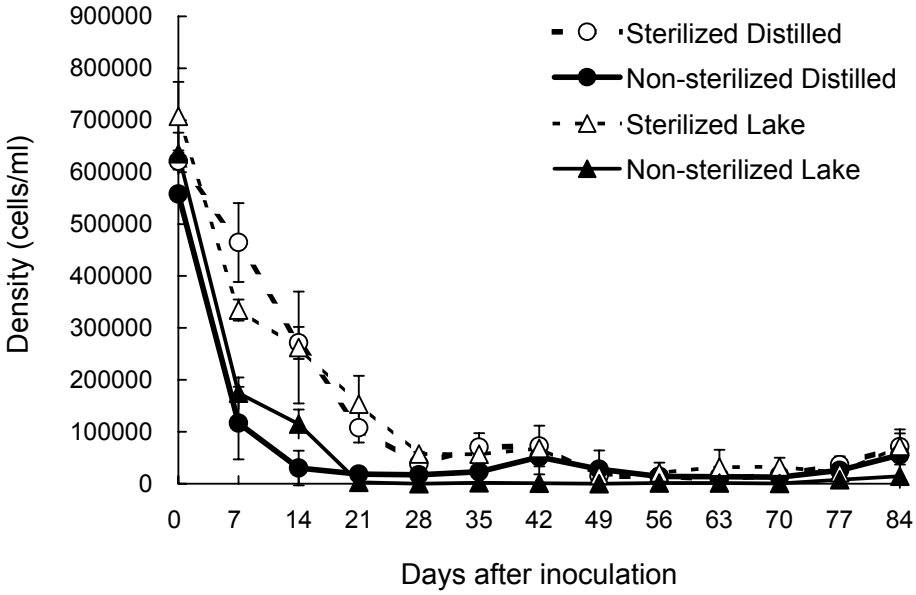

Fig. 1. Dynamics of densities of *Beauveria bassiana* in four kinds of waters by terms of CFUs. Vertical bars = SD.

and between sterilized and non-sterilized lake waters (two-way Repeated-Measures ANOVA, $p<0.0001$ for waters and $p<0.0001$ for days × waters).

Germination dynamics of *B. bassiana*

In the experimental waters, *B. bassiana* conidia produced germ tubes, hyphal bodies, and hyphae (Fig. 2). Newly reproduced conidia from hyphae or from hyphal bodies were also observed within a short time (Fig. 2). Among all the fungal cells, conidia with germ tubes, hyphal bodies, and hyphae were treated as germination. The newly reproduced conidia were obviously a consequence of germination, but they were not morphologically distinguishable from the ungerminated ones. In the first few weeks, germ tubes, hyphal bodies, and hyphae could be seen in all experimental waters, although their rates were not so high. Especially, visible cotton-like mycelia appeared and remained visible for a long time in the sterilized waters (Fig. 3), while such visible mycelia did not appear and

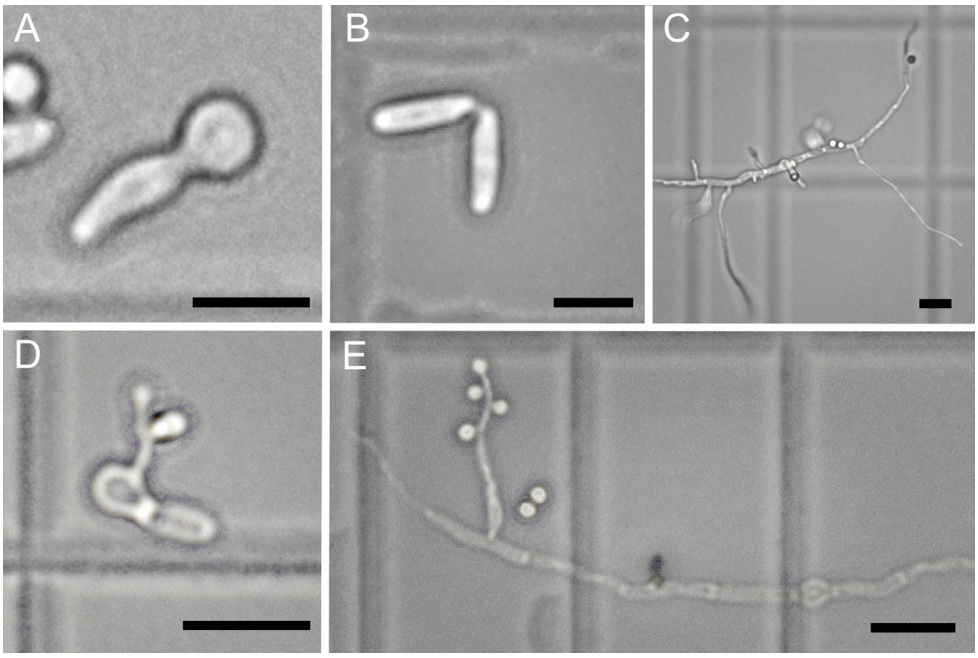

Fig. 2. Various morphologies as outcomes of germination of *Beauveria bassiana* conidia (A: elongation of germ tube, scale bar = 5 μm; B: hyphal bodies, scale bar = 5 μm; C: hyphae, scale bar = 10 μm; D: secondary reproduced conidia from a hyphal body, scale bar = 5 μm; E: secondary reproduced conidia from a hypha, scale bar = 10 μm).Fig. 1. Dynamics of densities of *Beauveria bassiana* in four kinds of waters by terms of CFUs. Vertical bars = SD.

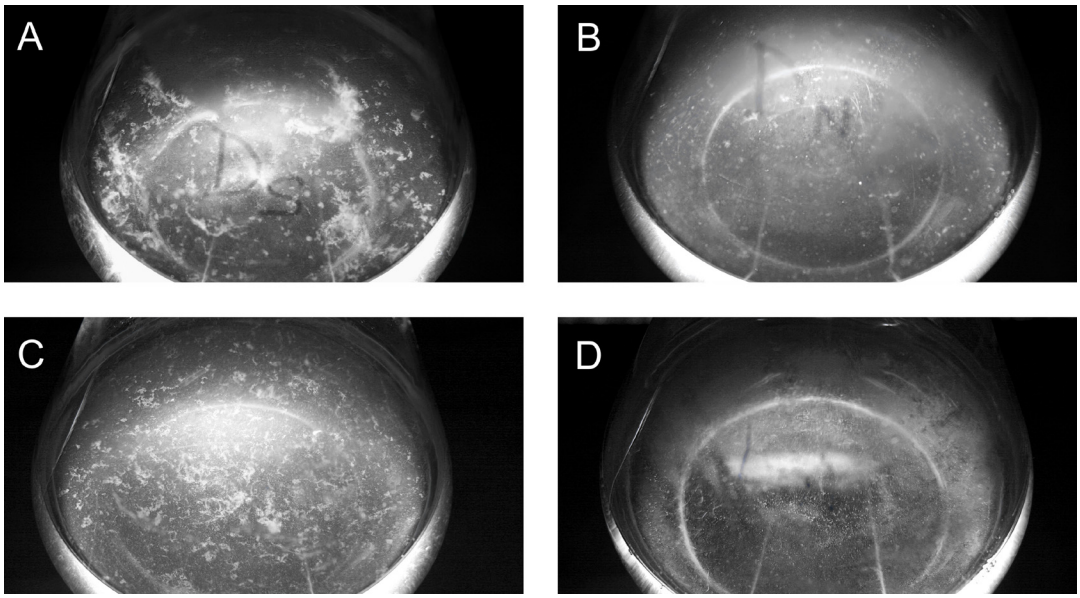

Fig. 3. Visible difference of the growth of mycelia as appeared in sterilized and non-sterilized groups. Some fuzzy cotton-like mycelia can be seen in sterilized waters (A: sterilized distilled water; B: non-sterilized distilled water; C: sterilized lake water; D: non-sterilized lake water).

the conidia were restrained from germination in both of the non-sterilized waters. The germination rates of *B. bassiana* conidia persisted in sterilized distilled water and sterilized lake water and ranged from 1.76% to 6.95%, and from 1.55% to 7.49%, respectively, within 48 d. In the next 5 weeks, the percentage of germinating conidia in the two groups rose and ranged from 10.45% to 18.08%, some higher than in the first half periods (Fig. 4). The germination rates in both of the non-sterilized waters were 0% after 27 d. Dynamics of germination rates in sterilized and non-sterilized distilled water differed significantly (two-way Repeated-Measures ANOVA, p=0.0072 for waters and p<0.0001 for days × waters). Those in sterilized and non-sterilized lake water also differed significantly (two-way Repeated-Measures ANOVA, p<0.0001 for waters and p<0.0001 for days × waters). On the other hand, those in non-sterilized distilled and lake water were not statistically different (two-way Repeated-Measures ANOVA, p=0.1855 for waters and p=0.2281 for days × waters respectively). Similarly, no statistical difference was found between those in sterilized distilled and lake water (two-way Repeated-Measures ANOVA, p=0.9856 for waters and p=0.3315 for days × waters respectively).

Discussion

Beauveria bassiana can infect many species of insects and can grow on artificial media or some kinds of soil (Li, 1988). It is believed that these hosts and survival niches supplied the necessary nutrients for growth (Smith & Grula, 1980). No report has been found studying whether *B. bassiana* could grow in water. The present study revealed that *B. bassiana* could germinate and extend hypha in sterilized waters, both in sterilized lake water and in sterilized distilled water. Just one-week after inoculation, visible long mycelia appeared in the two kinds of sterilized waters, but not in non-sterilized ones. The new mycelia showed degradation after 4 weeks survival; perhaps because of exhaustion of nutrients stored in the conidia. No visible mycelium appeared in non-sterilized waters, suggesting the influence of inhibition by other aquatic organisms.

The fungal densities in sterilized treatments were generally higher than in non-sterilized treatments. This phenomenon was especially conspicuous during the first one month of survival, but in the following several months, their densities were around 1 x 10^4 /ml except non-sterilized lake water in which the fungal density was almost 1/10 of that in other waters. Non-sterilized water must contain more microorganisms than distilled water, and they might keep the density of *B. bassiana* lower than in the other waters.

Beauveria bassiana persists in some niches including insect cadavers and soil (Wang et al., 2005). Wild *B. bassiana* could be isolated from natural soil (Doberski & Tribe, 1980; Shimazu et al., 2002a), and introduced populations of *B. bassiana* could keep a certain density for a long time in soil (Shimazu et al., 2002b; Wang et al., 2002). *Metarhizium anisopliae*, another important entomopathogenic fungus, could also survive for a long time in soil (Mikuni et al., 1982; Yaginuma, 1990). The present study revealed that *B. bassiana* could persist for relatively long periods (12 weeks) in sterilized waters, while in non-sterilized waters the fungus decreased sharply at the

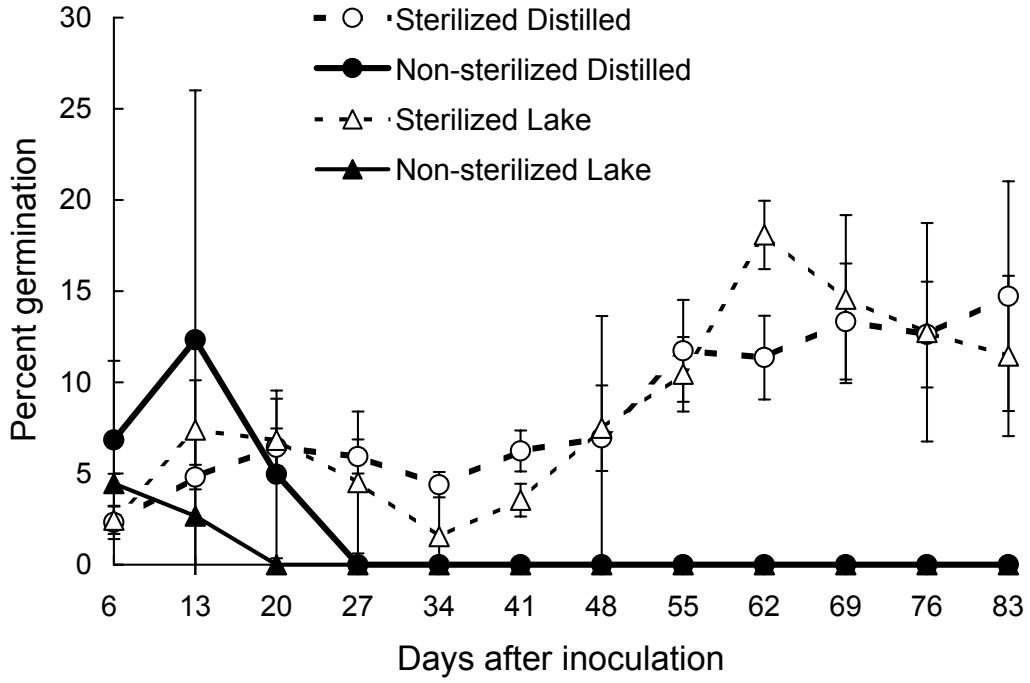

Fig. 4. Dynamics of conidial germination rates in four kinds of waters. Vertical bars = SD.

beginning and then remained at a low density.

Biotic factors in soil are disadvantageous to the growth of entomogenous fungi (Walstad et al., 1970; Pereira et al., 1993). Shimazu et al., (2002b) observed that *B. bassiana* conidia germinated in sterilized soil, but not in non-sterilized soil. Walstad et al. (1970) revealed that microbes in soil were detrimental to the growth and infection of *B. bassiana* to the pales weevil, *Hylobius pales*. Pereira et al. (1993) also found soil antagonism affecting the dose-response of workers of the red imported fire ant, *Solenopsis invicta*, to *B. bassiana* conidia. The half lethal concentration (LC50) increased from 1×10^2 conidia/g soil for sterilized soil to 2×10^9 conidia/g for non-sterilized soil. A similar result was found in water in the present experiment. In non-sterilized groups, germination of conidia was possibly inhibited by some microbes in water. Sterilization of water probably removed either microbial competition or inhibition to *B. bassiana*, and benefited the persistence of the fungus. Compared with soil, nutritional conditions in water are thought to be poorer. That may limit the germination of *B. bassiana* in sterilized water, and keep it at a low level. The conidia may quickly lose their viability when they exhausted their own stored nutrients.

Although *B. bassiana* has been known as a pathogen of many insect species, there have been few records from aquatic insects. Clark et al. (1968) reported *B. bassiana* conidia floated on the surface of the water and killed *Culex pipiens* larvae, but the virulence was not clarified. Miranpuri and Khachatourians (1991) found that both conidia and blastospores (=hyphal bodies) of this fungus had larvicidal activities against *Aedes aegypti* at concentrations of 4×10^8 cells/ml, although their infection route might not be percutaneous. The rice water weevil, Lissorhoptrus oryzophilus was highly susceptible to *B. bassiana* at a conidial concentration of 1×10^8/ml, and 5×10^{11}/m^2 of conidia were necessary to reduce the population density of the larvae and the pupae on rice plants to 30% of that of non-treated rice (Yoshizawa, 1998). Throughout those studies, numerous conidia are thought to have been needed to allow infection of aquatic insects compared with the susceptibility of *M. alternatus* larvae; whose LC_{50} to *B. bassiana* F-263 was 1.1×10^3/ml (Shimazu, 1994). Moreover, our isolate is expected to be used on dead pine logs in the forest and there will be a very low possibility for the conidia to mingle in water at high densities to infect aquatic insects.

B. bassiana F-263 does not seem to cause damage to aquatic insects for the following reasons when the fungus is applied to control *M. alternatus* in the forest; 1) large decrease of viable conidia in natural waters, 2) requirement of high dose to infect aquatic insects, and 3) low possibility of conidia to contaminate aquatic systems. Also, there is a concern about the infection of terrestrial insects by this fungus which may survive in water. However, this possibility should be far less than direct infection from applied conidia.

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昆虫病原菌 *Beauveria bassiana* の淡水中における密度動態

王濱 [2)]・島津光明 [1)*]

要 旨

Beauveria bassiana が水域生態系に影響をおよぼす可能性を検討するため，この菌の淡水中での密度変動を調査した。室内で *B. bassiana* の分生子を無殺菌湖水，殺菌湖水，無殺菌蒸留水，殺菌蒸留水に入れ，その密度を追跡した。いずれの水の中でも分生子は短時間に急速に減少した。28 日以内に 90％以上の分生子が消失し，とくに無殺菌湖水中では 0.03％しか生き残らなかった。無殺菌の水の中では 27 日以降には発芽している分生子はみられなくなったが，殺菌した水の中では接種後 84 日までみられた。自然の水の中では，この菌は存在している他の微生物により発芽を阻害され，生きた分生子の密度が減少したと考えられた。この結果から，偶然 *B. bassiana* が自然の淡水系に混入しても，増殖することは困難なことが明らかになった。

キーワード：密度，昆虫病原菌，水域生態系，発芽，分生子

＊森林総合研究所森林昆虫研究領域　〒 305-8687　茨城県つくば市松の里 1　e-mail: shimazu@ffpri.affrc.go.jp
1) 森林総合研究所森林昆虫研究領域
2) 中華人民共和国　安徽農業大学虫生菌研究中心（現在）

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Density-dependence and within-host competition in a semelparous parasite of leaf-cutting ants

William OH Hughes*[1,3], Klaus S Petersen[1], Line V Ugelvig[1], Dorthe Pedersen[1], Lene Thomsen[2], Michael Poulsen[1] and Jacobus J Boomsma[1]

Address: [1]Department of Population Biology, Institute of Biology, University of Copenhagen, Universitetsparken 15, 2100 Copenhagen, Denmark, [2]Department of Ecology, The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark and [3]School of Biological Sciences, A12, University of Sydney, Sydney, N.S.W. 2006, Australia

Email: William OH Hughes* - whughes@usyd.edu.au; Klaus S Petersen - KSPetersen@bi.ku.dk; Line V Ugelvig - l_ugelvig@hotmail.com; Dorthe Pedersen - kortedorthe@hotmail.com; Lene Thomsen - lthomsenaxelhoej@hotmail.com; Michael Poulsen - mpoulsen@bi.ku.dk; Jacobus J Boomsma - jjboomsma@bi.ku.dk

* Corresponding author

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Abstract

Background: Parasite heterogeneity and within-host competition are thought to be important factors influencing the dynamics of host-parasite relationships. Yet, while there have been many theoretical investigations of how these factors may act, empirical data is more limited. We investigated the effects of parasite density and heterogeneity on parasite virulence and fitness using four strains of the entomopathogenic fungus, *Metarhizium anisopliae* var. *anisopliae*, and its leaf-cutting ant host *Acromyrmex echinatior* as the model system.

Results: The relationship between parasite density and infection was sigmoidal, with there being an invasion threshold for an infection to occur (an Allee effect). Although spore production was positively density-dependent, parasite fitness decreased with increasing parasite density, indicating within-host scramble competition. The dynamics differed little between the four strains tested. In mixed infections of three strains the infection-growth dynamics were unaffected by parasite heterogeneity.

Conclusions: The strength of within-host competition makes dispersal the best strategy for the parasite. Parasite heterogeneity may not have effected virulence or the infection dynamics either because the most virulent strain outcompeted the others, or because the interaction involved scramble competition that was impervious to parasite heterogeneity. The dynamics observed may be common for virulent parasites, such as *Metarhizium*, that produce aggregated transmission stages. Such parasites make useful models for investigating infection dynamics and the impact of parasite competition.

Background

In most models of host-parasite dynamics, the parasites are considered as part of discrete infections, involving only a single parasite. However, as the transmission stages of parasites tend to be clustered, the majority of host-parasite interactions will rather involve multiple parasite individuals. This is particularly the case for those parasites that exhibit a semelparous life-history, releasing all their transmission propagules in a single event that normally coincides with host death [1,2]. In the same models, transmission is assumed to follow the mass action principle, βSI, where S and I are the densities of susceptible and infected individuals respectively and β is a constant describing the probability of infection [3]. Thus the probability of a host becoming infected per unit time will be directly proportional to the number of parasites that it encounters. Host-parasite interactions will also often involve multiple genotypes of parasites, further complicating their dynamics [4,5].

The co-occurrence of multiple parasites within a single host makes within-host competition between the parasites inevitable. Hosts represent limited resources and there is a carrying capacity for the total biomass of parasites that the host resources can support. The within-host growth of the parasites will thus normally be density-dependent, decreasing as the carrying capacity is approached [6,7]. Interactions between different parasite genotypes are often considered to result in increased virulence, but have also been predicted to lead to decreased virulence depending on the dynamics [4,8-11]. The outcome of interactions will depend critically on both the scale of competition and the relatedness of the parasites involved, with higher relatedness selecting for reduced competition, or even cooperation, and more local competition offsetting this [12-14]. The nature of the within-host competition has further been characterized as a continuum between two extremes: superinfections, which exhibit contest competition with the most virulent parasites eliminating those less virulent, and coinfections, where the parasites differ little in virulence and resources end up being shared amongst individuals via scramble competition (or in its purest form by parasites exploiting different within-host niches and not competing at all) [8,15,16]. The mechanism of competition can be: (1) exploitation, with parasites competing for resources, (2) interference, with parasites, for example, producing antagonistic compounds, or (3) apparent, being mediated by the host immune system [5]. Exploitation competition is inevitable whenever parasites do not have completely separate niches within the host, while interference competition via antagonistic compounds drives, for example, the interaction between strains of entomopathogenic bacteria that produce bacteriocins [17]. Apparent competition has been argued to be the most important type [4], and can occur even in invertebrates with their less complex immune systems. For example, malaria parasites suppress the immune response of their mosquito vector [18], and various entomopathogenic fungi have been shown to produce immunodepressant compounds [19-23].

In spite of the fundamental importance of interactions between different strains of parasites, empirical studies of mixed infections are relatively rare and a number of them have produced results that conflict with the theoretical predictions [5,24]. Most studies have found either that mixed infections are more virulent than single infections [25-29], or that virulence equalled that of the most virulent strain [23,25,30-32]. Interactions can be more complex though, and may also depend on environmental conditions, host genotype, or on the parasite genotypes involved [25,32-35]. For example, the virulence of bacteriocin-producing bacteria matches the winning strain only when one strain can kill the other, but is reduced compared to single infections when both strains can kill each other [17]. Furthermore, consideration of virulence only does not provide a full picture of the complexity of the interaction. The production of transmission stages may be increased [26-28,30] or decreased [31] during mixed infections, and may also depend upon the order of infection [32]. Although the finding that virulence matched that of the most virulent strain might suggest that that strain has outcompeted the less virulent strain, in some studies the transmission stages produced were from the both strains [29,31,33], or even entirely from the less virulent strain [23].

Here we examine the infection dynamics of the parasite *M. anisopliae* var. *anisopliae* (Metschnikoff) (Deuteromycotina: Hyphomycetes) in the leaf-cutting ant *Acromyrmex echinatior* Forel (Hymenoptera: Formicidae: Attini). *M. anisopliae* var. *anisopliae* is a generalist entomopathogenic fungus that is known to infect leaf-cutting ants [36-41], as well as many other insects. Infection may be from sporulating cadavers or from spores dispersed in the soil. *Metarhizium* spores have a tendency to remain attached to one another, making even dispersed spores likely to be clustered, and soil has been estimated to contain as many as 1,000 to 50,000 spores g^{-1} [41]. Spores germinate and penetrate directly through the host cuticle without first growing over the surface of it as some other fungi do. Host individuals will thus often be infected by multiple parasite propagules. In addition, the group-living life-style of leaf-cutting ants, as with other social insects, makes them especially prone to being exposed to multiple infections under natural conditions [42]. Inside the host, the parasite produces blastospores and then hyphal bodies that release immunodepressant and antibiotic toxins [19,21,22]. After a period of time the host dies by some combination of the depletion of its resources due to the

parasite infection, direct invasion of tissues by hyphae or the action of the parasite's toxins [21], and the parasite sporulates shortly after this. *Metarhizium* thus has a semelparous life-history and is an 'obligate killer' [1], producing transmission stages only after host death. Such parasites represent excellent models for studying infection-growth dynamics because the rate of successful infections equates exactly to host mortality, the time of host death will relate to the number of hyphal bodies within the host and thus acts as a gauge of within-host growth, and because the lifetime reproductive output of the parasite is represented entirely by the spores produced upon host death in contrast to other parasites that produce transmission stages continually [1]. Yet while applied studies using *Metarhizium* are common, fundamental studies of its infection dynamics are rare.

There is a diversity of *M. anisopliae* var. *anisopliae* strains near leaf-cutting ant nests in Panama [41], indicating the potential for within-host competition between multiple parasite genotypes. We established and compared the dynamics of within-host competition in this system at two levels. We first investigated competition between parasites of the same genotype by establishing whether the infection rate of *M. anisopliae* var. *anisopliae* adhered to the mass action principle and whether parasite growth and fitness was density-dependent. We then examined whether the infection dynamics were consistent between different strains of parasite, and how the dynamics were affected by intraspecific within-host competition involving multiple parasite genotypes. We used two Panamanian strains that had had the opportunity to coevolve with *A. echinatior* and one exotic strain that had never previously encountered the host. A number of studies suggest that exotic strains are less competitive during within-host interactions than are native strains that have coevolved with the host [43-48]. We define parasite virulence to be parasite-induced host mortality as measured by case mortality and time-of-death. This differs from the instantaneous mortality rate used in many models but has been argued to be a more suitable measure of virulence [49]. Note that because the ant hosts were adult individuals that do not grow any further, the potential advantage to an obligate killer parasite of delaying host death to allow further host growth before semelparous reproduction [1], will not apply in this system.

Results

Experiment 1: intra-strain competition

The dose of *Metarhizium anisopliae* var. *anisopliae* spores applied had a significant effect on ant mortality (Wald = 131.1, d.f. = 10, P < 0.0001) (Figure 1). The colony of origin did not affect either the dose-response relationship (Wald = 13.2, d.f. = 10, P = 0.213) or mortality overall (Wald = 1.56, d.f. = 1, P = 0.212). The mortality caused by the lowest two doses did not differ significantly from that in the controls (Figure 1). There was also no significant difference in mortality between the highest three doses because mortality was close to 100% at all these doses. The dose-mortality relationship consequently followed a sigmoidal pattern ($F_{3,7}$ = 162.5, P < 0.0001; Figure 2a).

There was no effect of the time between death and the assessment of spore production upon either the proportion of cadavers sporulating (Wald = 1.97, d.f. = 1, P = 0.16) nor the number of spores produced ($F_{1,127}$ = 1.13, P = 0.289). This supports the assumption that sporulation was complete at the time of assessment and also indicates that parasites that took longer to kill their host did not produce more spores. The proportions of cadavers sporulating were significantly less than expected based upon the number of ants estimated (from the control data) to have died from other causes ($F_{1,17}$ = 8.55, P = 0.0095). This indicates that certain ants killed by *Metarhizium* failed to sporulate. Both the proportion of ant cadavers sporulating (Wald = 19.4, d.f. = 9, P = 0.022) and the mean number of spores produced per sporulating cadaver ($F_{9,127}$ = 1.97, P = 0.048) increased significantly with dose (Figures 2b and 2c). However the increase in spore production was relatively small, only doubling over the full range of doses examined. When the spore numbers produced were used to calculate *k*-values (which assess the density-dependence of growth, with a zero value indicating that spore production increases proportionally to dose and positive values indicating spore production is less than proportional to dose), it was found that *k* increased significantly with dose ($F_{1,9}$ = 231.1, P < 0.0001; Figure 2d). The slope of the relationship (0.8) was significantly less than 1 (t = 2.42, d.f. = 8, P = 0.042). Correspondingly, the per capita fitness of the parasite decreased linearly with dose, while, in contrast, the probability of infection (calculated by multiplying the probability of death by the probability of a cadaver sporulating) increased sigmoidally (Figure 2e). By multiplying these two variables, the overall fitness of the parasite can be calculated, and can be seen to decrease more or less linearly with dose (Figure 2f).

Experiment 2: inter-strain competition

Ant survival was affected significantly by the concentration of *M. anisopliae* var. *anisopliae* spores (Wald = 186.0, d.f. = 5, P < 0.0001), the strain (Wald = 9.42, d.f. = 3, P = 0.024), and also the colony of origin (Wald = 13.5, d.f. = 4, P = 0.009). There were no significant interactions between strain and concentration (Wald = 11.8, d.f. = 15, P = 0.681). Mortality was generally positively correlated with dose, although the significance of pairwise differences between doses did vary somewhat between strains (Figure 3). In none of the strains was there any difference in survival between the lowest two doses, and, with the exception of strain 02–73, there was also no difference in

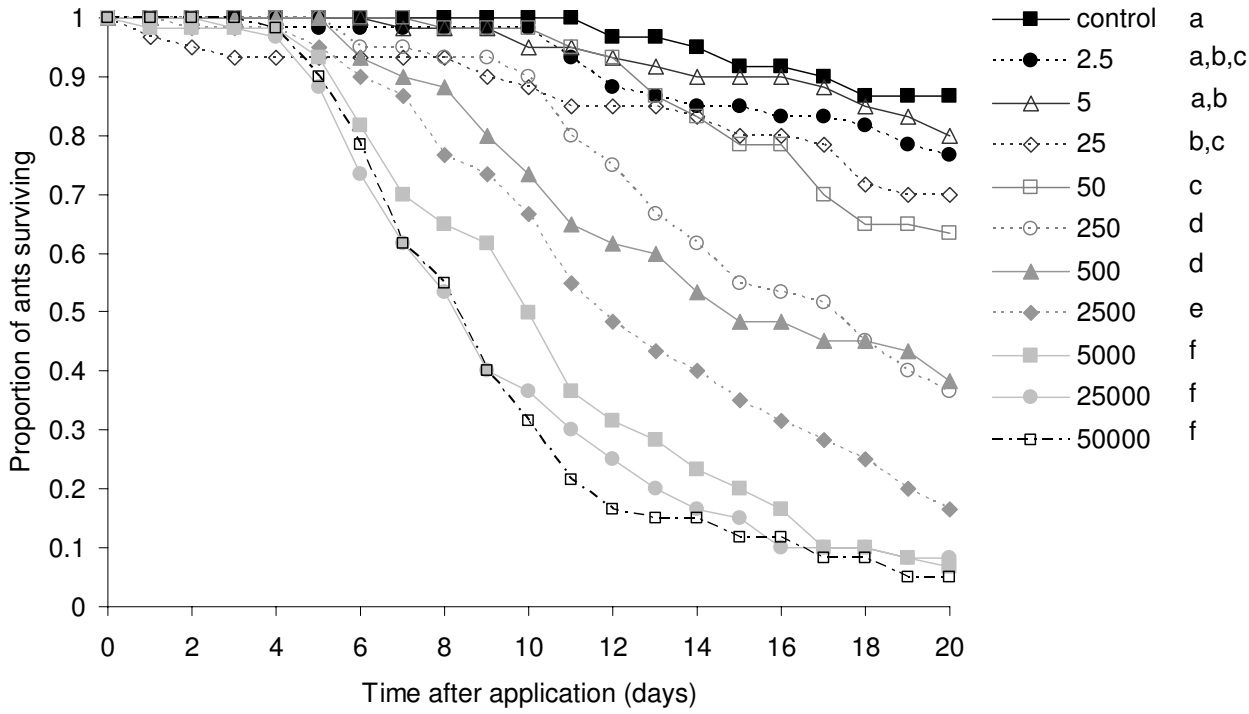

Figure 1
Survival of ants in Experiment 1 treated with serial doses (spores/ant) of *M. anisopliae* var. *anisopliae* isolate KVL 02–56 or a control solution of 0.05% Triton-X (n = 60). Different letters indicate doses whose survival distributions differed significantly.

survival between the highest two doses. Overall, ant survival was greatest when treated with the allopatric Ma275 strain, followed by the 02–73 strain (obtained from a soil sample at the collection site), from which it did not differ significantly (Breslow statistic = 2.45, d.f. = 1, P = 0.118). Survival of ants treated with Ma275 was significantly greater than of those treated with either the 02–72 strain (obtained from an *Atta* worker) (Breslow statistic = 8.36, d.f. = 1, P = 0.004) or the mixture of all three isolates (Breslow statistic = 5.00, d.f. = 1, P = 0.025) (Figure 3). The survival distributions of ants treated with the different strains did not otherwise differ significantly (02–72 vs. mixture: Breslow statistic = 0.84, d.f. = 1, P = 0.360; 02–73 vs. mixture: Breslow statistic = 0.26, d.f. = 1, P = 0.613; 02–72 vs. 02–73: Breslow statistic = 2.04, d.f. = 1, P = 0.153).

Although the proportion of ant cadavers sporulating was consistently high (>80%) in strain 02–73 and was positively correlated with dose in the other strains (Figure 4a), the interaction between strain and dose was nonsignificant (Wald = 8.92, d.f. = 15, P = 0.882), as were both the main effects (strain: Wald = 2.81, d.f. = 3, P = 0.422; dose: Wald = 5.49, d.f. = 5, P = 0.359). Spore production from the sporulating cadavers was estimated by the ranking method described. It was found to be unrelated to the lag-time between death and ranking ($F_{1,270}$ = 0.489, P = 0.485) and to increase slightly with dose in all treatments ($F_{5,270}$ = 2.42, P = 0.036; Figure 4b). The relationship between spore production and dose did not differ between strains ($F_{14,270}$ = 1.12, P = 0.342) and the strains also did not differ overall ($F_{3,270}$ = 0.19, P = 0.903). The *k*-values for all three strains were positively correlated with the number of spores applied ($F_{1,15}$ = 3612.9, P < 0.0001) and had similar slopes ($F_{3,15}$ = 0.78, P = 0.523; Figure 4c). The values for Ma275, though, were significantly lower than for the other strains ($F_{3,15}$ = 13.71, P = 0.0001).

Discussion

Parasite virulence, as measured by host mortality, was found to show a clear density-dependent pattern. This has also been recorded previously in *Metarhizium* [e.g. [50-53]] and other parasites [6,7,54]. Rather than being a linear relationship though, the pattern was sigmoidal. Mortality was not increased significantly by further increases in dose beyond 1×10^7 spores per ml. In addition, the

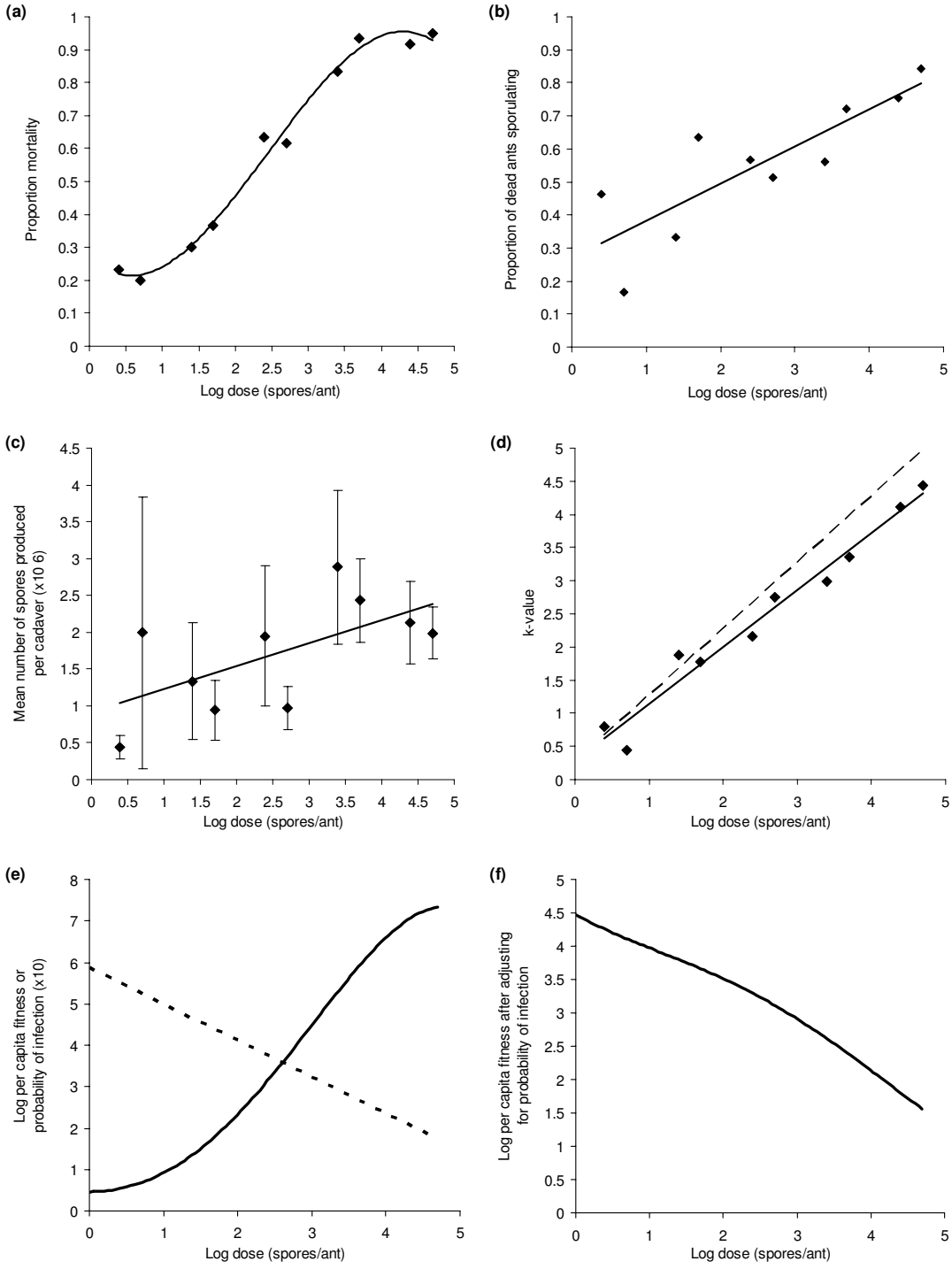

Figure 2
Dose relationships for ants in Experiment 1 treated with serial doses of *M. anisopliae* var. *anisopliae* isolate KVL 02–56 or a control solution of 0.05% Triton-X. (a) Mortality at end of experiment ($y = -0.021x^3 + 0.144x^2 - 0.054x + 0.171$, $r^2 = 0.986$). (b) Proportion of dead ants sporulating ($y = 0.113x + 0.269$, $r^2 = 0.700$). (c) Mean number of spores (± SE) produced per sporulating ant ($y = 0.3692x + 0.6692$, $r^2 = 0.4081$). (d) *k*-values for ants in Experiment 1 treated with serial doses of *M. anisopliae* var. *anisopliae* isolate KVL 02–56 ($y = 0.809x + 0.468$, $r^2 = 0.976$). The dashed line has a slope of 1 and is included for comparison. (e) Per capita fitness (dashed line) and probability of infection (solid line; calculated by multiplying the probability of death and of sporulation if death occurs). (f) Per capita fitness after adjusting for the probability of infection.

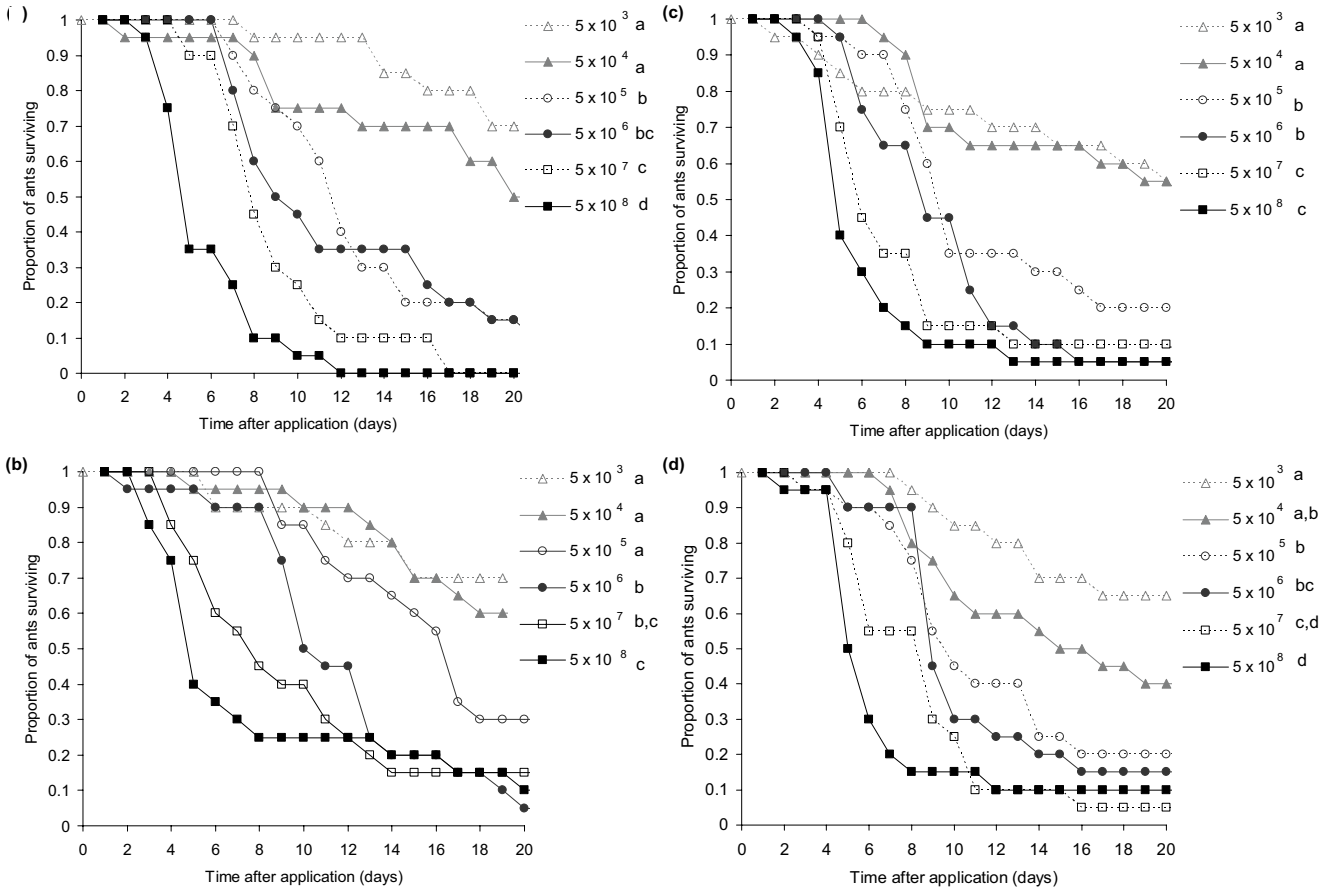

Figure 3
Survival of ants in Experiment 2 treated with either *Metarhizium anisopliae* var. *anisopliae* isolate (a) KVL 02–73, (b) KVL 02–72, (c) Ma275 or (d) a mixture of all three. Different letters indicate doses whose survival distributions differed significantly.

mortality caused by doses of 1×10^4 spores per ml or less did not differ from that of ants treated with the control solution (Figure 2a). All parasite-induced mortality was expected to have occurred by twenty days after treatment, even at the lower doses, and this assumption was supported by the levelling out of mortality seen in both experiments. It follows from this that the infection rate of *Metarhizium* is directly represented by the host mortality rate after twenty days. The lack of a difference in mortality between ants treated with the two lowest doses and the control ants suggests the occurrence of an Allee effect, with an invasion threshold for infection to be successful [54]. Such an effect has also been evidenced in some other studies of *Metarhizium* [e.g. [50,53]], and is a subtle effect that will only be detected when a sufficient range of doses is tested. Many models of host-parasite dynamics are based upon the mass-action principle, under which the infection rate is a linear function of the density of parasites a host individual encounters [3]. The sigmoidal pattern observed in the *Metarhizium-Acromyrmex* system indicates that, here at least, this principle applies only at intermediate doses.

The Allee effect most probably relates to the effectiveness of the host defences against parasites. Leaf-cutting ant defences, as with most insects, consist of 'first-line' defences involving grooming and the secretion of antibiotic compounds on to the cuticle, and 'second line' defences based upon the cellular and humoral immune responses [37,40,55-58]. Spores will interact independently with the first line defences, the immune response may be saturated if it has to defend against very high numbers of parasites. This effect will be exacerbated by the toxins that the hyphae of many *Metarhizium* strains produce to incapacitate the immune system [19,21,22,59]. Greater doses of infecting spores will more quickly produce a larger pool of mycelium, which will produce greater quantities of these toxins and thus make the immune

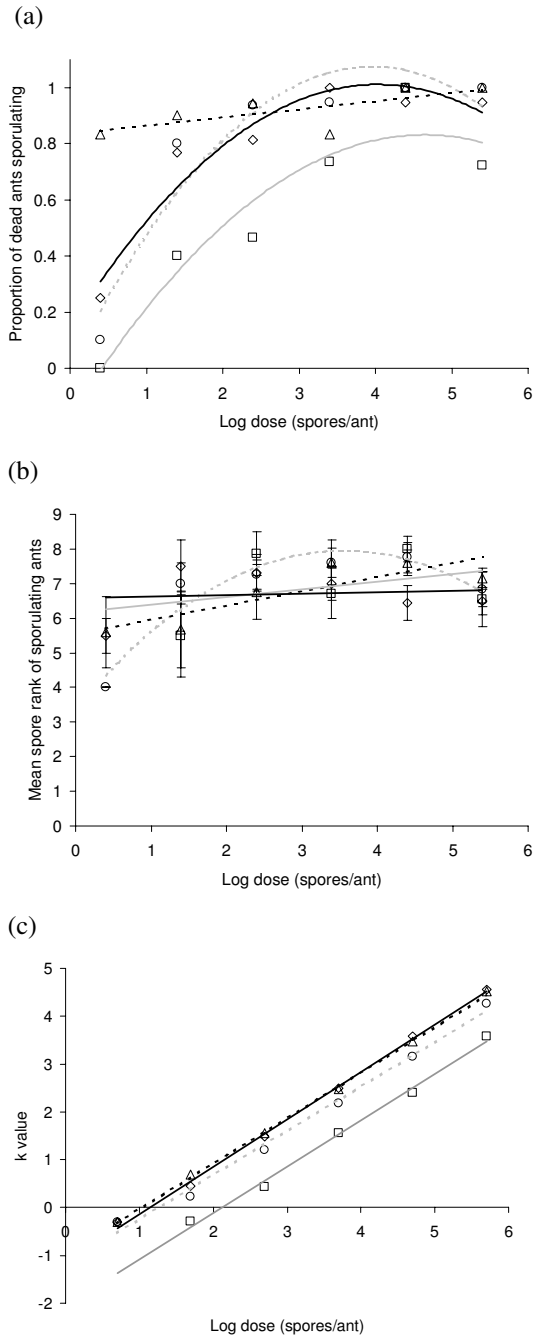

Figure 4
Dose relationships and lines of best fit for ants in Experiment 2 treated with either *Metarhizium anisopliae* var. *anisopliae* isolate KVL 02–72 (circles; grey, dashed line), KVL 02–73 (triangles; black, dashed line), Ma275 (squares; grey, solid line) or a mixture of all three (diamonds; black, solid line). (a) Proportion of dead ants sporulating. Lines of best-fit are: KVL02–72: $y = -0.0686x^2 + 0.5434x - 0.0014$, $r^2 = 0.8983$, P = 0.032; KVL 02–73: $y = 0.0292x + 0.8339$, $r^2 = 0.5182$, P = 0.107; Ma275: $y = 0.1623x + 0.0839$, $r^2 = 0.7699$, P = 0.030; mixture: $y = -0.0532x^2 + 0.4286x + 0.1476$, $r^2 = 0.9291$, P = 0.019. (b) Spore ranks of sporulating ants. Lines of best-fit are: KVL02–72: $y = -0.358x^2 + 2.5553x + 3.391$, $r^2 = 0.9148$, P = 0.025; KVL 02–73: $y = 0.411x + 5.5392$, $r^2 = 0.7212$, P = 0.032; Ma275: $y = 0.222x + 6.1677$, $r^2 = 0.116$, P = 0.575; mixture: $y = 0.1261x^3 - 1.2959x^2 + 3.7405x + 4.265$, $r^2 = 0.9646$, P = 0.833. (c) *k*-values. Lines of best-fit are: KVL02–72: $y = 0.93x - 1.18$, $r^2 = 0.992$, P < 0.0001; KVL 02–73: $y = 0.953x - 0.982$, $r^2 = 0.999$, P < 0.0001; Ma275: $y = 0.972x - 2.06$, $r^2 = 0.995$, P = 0.0002; mixture: $y = 0.991x - 1.127$, $r^2 = 0.998$, P < 0.0001.

system decreasingly capable of mounting an effective response.

Neither the probability of a cadaver sporulating nor the number of spores it produced were related to the time of host death, indicating both that parasites did not gain increased spore production by taking longer to kill the host, and that spore production was complete at the time of assessment. Both spore production and the proportion of cadavers sporulating were positively correlated with the within-host density of the parasite. However, the efficiency of the conversion of host biomass into parasite propagules was negatively density-dependent, as demonstrated by the *k*-values. The number of spores produced was less than proportional to increases in the dose of parasites applied, indicating the occurrence of density-dependent parasite growth and within-host competition. Although this competition was between genetically identical parasites in the first experiment, it still resulted in a reduction in parasite fitness because of the less efficient use of host resources. Were spore production to be unaffected by, or even inversely related to dose, as found in a study with *Beauveria* [60], then this negative effect on fitness would be even greater.

It is important to note that this is based only upon the cadavers that sporulated. As both the proportion of cadavers that sporulated and the number of ants that died increased with dose, there is a trade-off for the parasite (Figure 2e). Higher doses will result in more hosts dying and producing parasite spores, but where cadavers do sporulate, the per capita spore production will decrease as dose increases. Interestingly, this trade-off resolves itself such that increases in dose always result in a decrease in fitness (Figure 2f). Although low numbers of parasite spores have only a small chance of successfully infecting a host, the strong effect of within-host competition makes dispersal the best strategy. This is even without taking into account the probability of at least one spore encountering a host, which will also be substantially increased by spores dispersing (and thus having multiple chances to encounter a host per unit time), rather than staying in a single aggregation (and having only a single chance per unit time to encounter a host). In other studies of obligate killer parasites, even stronger effects of within-host competition between propagules of the same parasite clone have been found [7,60]. It therefore seems likely that these dynamics may be broadly similar for most semelparous, obligate killer parasites, and that maximum dispersal is the best strategy for these parasites, as well as any others that exhibit strong within-host competition.

When the different strains of *M. anisopliae* var. *anisopliae* were compared in the second experiment, they were found to differ in their virulence. Interestingly, it was the exotic strain (Ma275), originating from a different geographical location and from a different host order (Lepidoptera) that had the lowest virulence, and the strain isolated from a leaf-cutting ant worker (KVL 02–72) that had the highest. Many studies have found *Metarhizium* strains to vary in virulence [e.g. [53,61]]. Differences in strain virulence may in part be due to the variation in the production of destruxins that occurs between strains, but other virulence factors are undoubtedly also important [59]. As virulence is defined here as parasite-induced host mortality, the differences between strains could also be due to differences in the proportions of spores germinating and penetrating into the host rather than differences in within-host growth [62,63]. Although all strains had similarly high germination rates on artificial media, the interaction with host cuticle is more complex and involves various antibiotic compounds such as those produced by the metapleural gland [56,64,65]. Differences between strains in their susceptibility to such compounds would seem quite likely. Aside from the differences in virulence, however, the strains did not otherwise differ in their infection dynamics. They showed similar spore production and identical density-dependent growth patterns. The dynamics described above therefore appear to be consistent across strains, at least for those tested here.

A fundamental assumption of most models of host-parasite relationships is that within-host competition is more intense, and results in heightened virulence, when it involves more than one parasite genotype [8-10]. However, in experimental studies virulence has often been found to be unaffected by parasite heterogeneity [24]. This was also the case in the current study, in which the virulence of the mixed infection was the same as that of the most virulent strain in the infection. In addition, the parasite *k*-value vs. dose relationship was identical for the mixed and single infections, indicating that the density-dependent growth patterns were unaffected by host heterogeneity.

There are two possible explanations for the results. The most parsimonious is that the most virulent strain in the mixed infections simply outcompeted the other strains and drove them to extinction within the host. Such superinfection dynamics have previously been suggested for *Metarhizium* and other entomopathogenic fungi [32,66,67] and would be in accord with some models [6]. However other studies have found mixed infections to produce transmission stages from more than one strain of parasite in spite of the overall virulence matching that of the most virulent strain [29,31]. It remains possible that the mixed infections in the current study did not involve any of the strains being competitively excluded and that the spores produced came from all the strains. To distinguish between these possibilities it would be necessary to

isolate and sequence monospore cultures from the sporulating cadavers, something would be an interesting objective for future work.

Importantly, the infection dynamics did not differ between the single and mixed infections. The occurrence of within-host competition, whether it involves the production of antagonistic compounds or is mediated by the host's immune system, might be expected to force parasites to divert some resources from growth and the production of transmission stages to producing or coping with competitive mechanisms. While there is some evidence for this [31], the production of transmission stages has been increased during mixed infections in many other studies [26-28,30]. Clearly the outcome will depend upon the particular genotypes involved. If different strains produce antagonistic compounds that are effective against one another, as in the study by Massey et al. [17], then reduced transmission stages can be expected. If they do not, and if they exploit different within-host niches, produce cooperative compounds (such as iron-binding agents [13,14]), or act synergistically to depress the host immune system, then increased transmission stages can be expected. The fact that spore production was the same in single and mixed infections in the current study therefore suggests either that the most virulent strain outcompeted the others without suffering any cost from the competitive interaction, or that the *Metarhizium* strains were engaged in scramble competition with dynamics that are impervious to interactions being inter- or intraclone.

Conclusions

The importance of within-host competition between parasites is well illustrated by the *Metarhizium-Acromyrmex* system studied here. Even though the probability of a successful infection was increased substantially by parasites occurring in aggregations, the effect of competition between parasite propagules of the same clone makes dispersal the best strategy. It seems likely that this is generally true for semelparous parasites. Further investigations of the impact of within-host competition on parasite fitness are needed and should endeavour to establish where on the superinfection-coinfection continuum the interaction lies by identifying which parasites produce transmission stages. The fact that the production of transmission stages of semelparous parasites, such as *Metarhizium*, is concentrated into a single bout, and thus that their lifetime fitness can be readily quantified, makes them excellent models for doing this.

Methods

General methodology

Colonies of *A. echinatior* were collected from Gamboa, Panama, and maintained in the lab under controlled conditions (ca. 24°C, 70% RH) on a diet of bramble leaves (*Rubus fruticosus*) and rice grains. For the experimental replicates, large workers (head width 2.1 to 2.4 mm) were removed from their colonies and placed individually in plastic pots (diameter: 2.5 cm, height: 4 cm) where they were maintained at 24°C with an *ad libitum* supply of water and sugar water. A number of isolates of *M. anisopliae* var. *anisopliae* were collected from the vicinity of leaf-cutting ant nests at the same location in Gamboa, Panama, and were cultured as monospore isolates on Sabouraud dextrose agar [41]. Spore (conidia) suspensions were made by flooding agar plates with mature spores with a sterile solution of 0.05% Triton-X and scraping off the spores with a glass rod. The spores were centrifuged and washed three times with sterile 0.05% Triton-X solution with intervening centrifugation steps. The concentration of spores was then quantified using a haemocytometer and diluted to the required concentration. The viability of the spore suspensions was checked by spreading 100 μl of them on to Sabouraud dextrose agar plates and counting the proportions of spores that had germinated after 12–16 hours at 24°C. Spore viability was >95% in all cases.

Ants were treated with *M. anisopliae* var. *anisopliae* by applying 0.5 μl of a spore suspension to their thorax using a micropipette. Spore suspensions were vortexed thoroughly immediately prior to application to ensure spores were fully dispersed. Control ants had 0.5 μl of a 0.05% Triton-X solution applied in the same way. Following application, ant mortality was assessed daily for a period of twenty days. Based on previous work [23,40,56], this time period was judged sufficient to ensure that all parasite-induced mortality had occurred by the end of the experiment. Dead ants were surface sterilised [68], and placed in a petri dish lined with damp filter paper. After the completion of the experiments, the cadavers were left for a further ten days in order to allow full sporulation of the parasite. The level of sporulation on the cadavers was then assessed by one of two methods. In Experiment 1, sporulation was quantified by directly counting the number of spores on the cadavers. The cadavers were placed in individual vials with 1 ml of 0.05% Triton-X solution and vortexed for 1 min to remove the spores into suspension. The concentration of spores in the suspension was then quantified with a haemocytometer. In Experiment 2, sporulation was estimated by examining the cadavers under a binocular microscope and giving each a rank of between 0 (no spores visible) and 10 (cadaver almost completely covered by spores) depending upon the level of sporulation. Based on data collected prior to these experiments, spore ranks estimated in this manner correlate well with the actual number of spores on the cadavers ($y = 0.159x - 0.862$, $r^2 = 0.746$, Spearman's r = 0.820, N = 73, P < 0.01) and thus provide a reliable estimate of spore production.

Experiment 1: intra-strain competition

The experiment involved *M. anisopliae* var. *anisopliae* isolate KVL 02–56, which had been isolated from the dump pile of an *Atta colombica* nest in Gamboa, Panama [41]. A spore suspension was made up and serially diluted fivefold to give concentrations from 1×10^8 to 5×10^3 spores ml^{-1} (equivalent to an average of 50,000 and 2.5 spores per ant respectively). Given that soil at the site in Panama has been estimated to contain as many as 1,000 to 50,000 spores g^{-1} [41], it seems likely that this range encompasses the natural doses that the ants are exposed to. Thirty ants from each of two colonies of *A. echinatior* (Ae47 and Ae109) were treated with each of these doses, or with the control solution, and their survival monitored for twenty days after application. The effect of dose and colony of origin on ant mortality was analysed with a Cox proportional hazard regression model to examine the effect of parasite density (dose) on virulence and infection rate. This incorporates both case mortality and the time of death. Pairwise comparisons of the doses were done using Kaplan-Meir survival analyses and the Breslow statistic. The ant cadavers were left for ten days after the end of the experimental period, in order to allow ample time for all cadavers to sporulate fully. The numbers of cadavers sporulating and the numbers of spores produced by these cadavers were assessed with binary logistic and general linear models respectively. These data were used to calculate *k*-values to assess whether parasite growth was density dependent, as done previously by Ebert et al. [7]:

$$k_i = \log_{10} (M_0 D_i / M_i D_0)$$

where k_i is the *k*-value at dose *i*, *M* is the number of spores produced, *D* is the number of spores applied, and M_0 and D_0 represent the number of spores produced and applied respectively at the lowest dose tested. A zero value for *k* indicates that the number of spores produced at dose *i* are exactly proportional to the increased dose (so, for example, a doubling of dose results in a doubling of the number of spores produced). The equation gives a positive value for *k* when the number of spores produced at dose *i* are less than proportional to the increase in dose. Zero values for *k* thus indicate that spore production is independent of parasite density, while positive values of *k* indicate that spore production is negatively density dependent.

Experiment 2: inter-strain competition

To establish whether the dynamics recorded in the first experiment were consistent for different strains of the parasite, three isolates of *M. anisopliae* var. *anisopliae* were compared. These were KVL 02–73 (isolated from soil at the field site from which the ant colonies were collected), KVL 02–72 (isolated from an *Atta colombica* leaf-cutting ant worker), and the strain Ma275 (isolated from *Cydia pomonella* (Lepidoptera: Tortricidae) in Germany). The strains therefore represented a range in terms of their potential coevolution with *A. echinatior*, with the former two being likely to have had some interaction while Ma275 would not previously have encountered *A. echinatior*. Each strain was diluted tenfold and tested at six doses from 5×10^8 to 5×10^3 spores ml^{-1}. In addition, a mixed spore suspension was made up to examine inter-strain competition. The suspension contained equal numbers of spores of each of the three strains. This was serially diluted and applied at the same doses as the individual isolates. Four ants from each of five colonies of *A. echinatior* (Ae48, 109, 143, 153 and 154) were treated with each dose of each isolate or with the control solution and their survival monitored for twenty days. The survival of the ants was analysed as in the preceding experiment to examine if strains differed in virulence and if the growth of each strain was density-dependent. Ten days after the end of the experimental period, spore production was assessed by ranking each cadaver for the amount of sporulation as described earlier. These ranks were used to estimate the actual number of spores produced and these values were then used to calculate *k*-values. A regression analysis was carried out on these data in order to assess if the growth of each strain was density-dependent and whether the density-growth dynamics differed between strains.

Authors' contributions

WOHH conceived the study, assisted with the experiments, analysed the results, and drafted and revised the manuscript. KSP, LVU and DP carried out Experiment 1 and KSP also participated in Experiment 2. MP assisted with both experiments. LT assisted with mycological aspects and JJB provided support throughout. All authors contributed to the writing of the manuscript.

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BIOPESTICIDES REGISTRATION ACTION DOCUMENT

Beauveria bassiana strain ANT-03

Pesticide Chemical (PC) Code: 129990

U.S. Environmental Protection Agency
Office of Pesticide Programs
Biopesticides and Pollution Prevention Division

March 30, 2015

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Joel V. Gagliardi, Ph.D.	Product Analysis and Human Health
In-soon You, Ph.D.	Environmental Effects

Regulatory Review

Shannon Borges	Team Leader
ShaRon Carlisle	Acting Associate Chief
Denise Greenway	Regulatory Action Leader
Kimberly Nesci	Chief

I. EXECUTIVE SUMMARY

Background

In April 2013, Technology Sciences Group, Inc., on behalf of Anatis Bioprotection Inc., submitted an application for a new manufacturing-use pesticide product containing the new active ingredient *Beauveria bassiana* strain ANT-03 at 100.0% concentration in powder form (EPA File Symbol 89600-R) to the United States Environmental Protection Agency (EPA), under section 3 of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). An application for a new end-use pesticide product containing the new active ingredient *Beauveria bassiana* strain ANT-03 at 20.0% concentration (EPA File Symbol 89600-E) was concurrently submitted.

Beauveria bassiana strain ANT-03 is a naturally occurring bacterium and was originally isolated in 2000 from *Lygus lineolaris* found dead in a field in Sherrington, Quebec, Canada. The natural occurrence of *Beauveria bassiana* strains located in soils and insects is well documented in the US and worldwide.

The EPA's scientists reviewed product analysis, toxicology, and non-target organism data and information (40 CFR §§ 158.2120, 158.2140, and 158.2150, respectively) submitted to support the registration of the proposed pesticide products. They found that overall, such data and information are adequate for risk assessment purposes, fulfill the current microbial pesticide data requirements, and support registration of the products under FIFRA section 3(c)(5).

Product Analysis

For the purposes of FIFRA section 3(c)(5) registration, the product analysis data requirements for the manufacturing-use and end-use pesticide products were fulfilled by acceptable guideline studies. These data requirements include product chemistry and composition, analysis of samples, and physical and chemical characteristics.

Toxicology

The EPA has determined that all applicable mammalian toxicology data requirements for *Beauveria bassiana* strain ANT-03 are fulfilled. Acceptable Tier I mammalian toxicology data and other information support the registration of the pesticide products and the request for an exemption from the requirement of a tolerance for residues of *Beauveria bassiana* strain ANT-03 in or on all food commodities. Tier II and Tier III studies were not required because of the lack of acute toxicity/pathogenicity in the Tier I studies.

Tolerance Exemption

In its application to the EPA in 2013, Technology Sciences Group, Inc., on behalf of Anatis Bioprotection Inc., provided a petition, data and other information to support an exemption from the requirement of a tolerance for residues of *Beauveria bassiana* strain ANT-03 when used in or on all food commodities. No comments were received following publication of this notice.

Non-Occupational Exposure

Given the home and garden proposed use pattern for *Beauveria bassiana* strain ANT-03, non-occupational exposure is expected. However, repeated exposures to *Beauveria bassiana* strain ANT-03 from pesticidal applications will not exceed the EPA's level of concern in light of the

available data that demonstrate *Beauveria bassiana* strain ANT-03 is not toxic, is non-irritating, and is not pathogenic when used as labeled in accordance with good agricultural practices.

Non-target Organisms

Guideline studies and waiver rationale were submitted to meet data requirements for non-target organisms for *Beauveria bassiana* strain ANT-03. These data and waiver rationale are sufficient to fulfill the relevant microbial pesticide data requirements and for risk assessment purposes. Further testing of non-target organisms at higher tier levels (i.e., Tiers II, III, and IV) is not required. The EPA's scientists made a "No Effect" determination for federally listed threatened and endangered mammals, birds, and terrestrial and aquatic plants. Effects to listed freshwater and marine/estuarine fish and invertebrates and insects could not be precluded, and a determination requires additional analysis. Adverse effects to all other non-target organisms are not expected to result from the registration of *Beauveria bassiana* strain ANT-03 when applied in accordance with the directions on the label.

Public Participation

On October 1, 2009, the EPA announced a policy to provide a more meaningful opportunity for the public to participate in major registration decisions before they occur. According to this policy, the EPA provides a public comment period prior to making a registration decision for, at minimum, the following types of applications: new active ingredients; first food uses; first outdoor uses; first residential uses; or any other registration actions for which the EPA believes there may be significant public interest.

Consistent with the policy of making registration actions more transparent, the proposed pesticide products containing *Beauveria bassiana* strain ANT-03, a new active ingredient, and allowing for this active ingredient's first food, outdoor, and residential uses were subject to a 15-day comment period. During this comment period, the EPA received no comments from the public.

Registration Decision

The EPA maintains that, based upon the risk assessment and information submitted in support of *Beauveria bassiana* strain ANT-03 Technical and Bioceres, it is in the best interest of the public and the environment to issue these registrations. The basis for this decision can be found in the risk assessment for *Beauveria bassiana* strain ANT-03, which is characterized throughout this Biopesticides Registration Action Document (BRAD) and the associated referenced documents.

II. ACTIVE INGREDIENT OVERVIEW

Biological Name: *Beauveria bassiana* strain ANT-03

Culture Collection: Deposited at the Agricultural Research Service Culture Collection (NRRL) under NRRL 50797

OPP Chemical Code: 129990

Type of Pesticide: Microbial Pesticide – Insecticide

See Appendix B for specific information (i.e., use sites, application rates, methods of application, formulation types, and target pests) regarding the pesticide products containing this active ingredient.

III. REGULATORY BACKGROUND

A. Applications for Pesticide Registration

On April 25, 2013, applications for registration were submitted on behalf of Anatis Bioprotection Inc., 278, rang Saint-Andre´, St.-Jacques-le-Mineur, Quebec J0J 1Z0, Canada for the manufacturing-use pesticide product, *Beauveria bassiana* strain ANT-03 Technical (EPA File Symbol 89600-R), and the end-use product, Bioceres (EPA File Symbol 89600-E), containing the new microbial active ingredient *Beauveria bassiana* strain ANT-03. In the Federal Register of December 11, 2013 (78 FR 75343), the EPA announced receipt of these applications for registration of a pesticide product containing a new active ingredient for manufacturing-use and end-use. No comments were received following the publication of this notice. For more information, see the following docket at *www.regulations.gov*: EPA-HQ-OPP-2013-0718.

B. Food Tolerance Exemption

Concurrent with its registration applications and under the Federal Food, Drug, and Cosmetic Act (FFDCA) section 408(d), Anatis Bioprotection Inc. submitted a petition to establish an exemption from the requirement of a tolerance for residues of the pesticide active ingredient, *Beauveria bassiana* strain ANT-03 [Pesticide Petition (PP) 3F8176], in or on all food commodities. The EPA published a Notice that Anatis Bioprotection Inc. filed a petition to establish an exemption of the requirement for a tolerance for residues of *Beauveria bassiana* strain ANT-03 in or on all food commodities in the Federal Register of December 30, 2013 (78 FR 79359) and opened a 30-day comment period. No comments were received following publication of this notice. For more information, see the following docket at *www.regulations.gov*: EPA-HQ-OPP-2013-0717.

On December 24, 2014, the EPA established an exemption from the requirement of a tolerance for residues of *Beauveria bassiana* strain ANT-03 in or on all food commodities, when applied as microbial insecticide and used in accordance with label directions and good agricultural

practices (40 CFR § 180.1328; 79 FR 77395).

C. Registration Review

In September 2011, the EPA completed a Final Work Plan for *Beauveria bassiana* strains, Registration Review Case 6057. The plan includes a discussion of the Endangered Species assessment that will be completed in advance of a Proposed Registration Review Final Decision. This active ingredient will now be considered as part of the *Beauveria bassiana* strains Registration Review Case 6057. For more information, see the following docket at *www.regulations.gov*: EPA-HQ-OPP-2010-0564.

The end-use product containing *Beauveria bassiana* strain ANT-03 is proposed for use on growing crops as a contact mycoinsecticide to control or suppress foliar-feeding pests and certain grubs. Several other strains of *Beauveria* bassiana, including strains 447, GHA, HF23 and ATCC 74040, have previously been registered by the EPA and have similar use patterns.

IV. RISK ASSESSMENT

For definitions of scientific terms, please refer to *http://www.epa.gov/pesticides/glossary/*.

In the Federal Registers of October 26, 2007 (72 FR 60988), and August 30, 2012 (77 FR 52610), the EPA issued Final Rules on the data requirements to support registration of microbial pesticides and updated the definition for microbial pesticides. The data and information evaluated for this BRAD were considered in light of these requirements.

The EPA classifies each data submission with an indication of the usefulness for risk assessment of the information contained in the documents. A rating of "acceptable" indicates the study is scientifically sound and is useful for risk assessment. A "supplemental" rating indicates the data provide some information that can be useful for risk assessment. The studies may have certain aspects determined not to be scientifically acceptable ("supplemental: upgradable"). If a study is rated as "supplemental: upgradable," the EPA always provides an indication of what is lacking or what can be provided to change the rating to "acceptable." If there is simply a "supplemental" rating, the reviewer will often state that the study is not required by 40 CFR Part 158. Both "acceptable" and "supplemental" studies may be used in the risk assessment process as appropriate. An "unacceptable" rating indicates that new data must be submitted.

For the acute toxicity data requirements, Toxicity Categories are assigned based on the hazard(s) identified from studies and/or other information submitted to the EPA in support of a pesticide registration. The EPA classifies the active ingredients or particular product into Toxicity Category I, II, III, or IV, where Toxicity Category I indicates the highest toxicity and Toxicity Category IV indicates the lowest toxicity (see section II.B. of U.S. EPA (2014c)).

A. Product Analysis Assessment (40 CFR § 158.2120)

Microbial pesticide product analysis data requirements include product chemistry and composition, analysis and certified limits, and physical and chemical characteristics data.

Product chemistry and composition data include information about the identity of the active ingredient, the manufacturing process, deposition of a sample of the microbial active ingredient in a nationally recognized culture collection, and discussion of the potential for formation of unintentional ingredients. Analysis and certified limits data include information on analysis of samples and certification of limits. Physical and chemical characteristics data describe basic characteristics of the registered pesticide products, including color, physical state, odor, stability, miscibility, pH, corrosion characteristics, viscosity and density.

For the purposes of registration under FIFRA section 3(c)(5), the EPA determined that all product analysis data requirements for registration of the *Beauveria bassiana* strain ANT-03 pesticide products have been fulfilled. The following terms of registration are imposed not due to risk concerns, but to clarify and define the quality assurance/quality control measures:

- A method for spore identification must be added to the manufacturing process;
- Batch analysis using viable count calculations must be submitted;
- Specific methods for microbial contamination analyses must be provided;
- Rejection limits for contaminants must be defined; and
- Unidentified fungi must be explained.

These data must be submitted within ninety days of the registration date, except that 12 months are alloted for receipt of the batch analyses.

A new, unique PC Code 129990 has been assigned to *Beauveria bassiana* strain ANT-03, as it is a new active ingredient. A sample of *Beauveria bassiana* strain ANT-03 is deposited at the Agricultural Research Service Culture Collection (NRRL) under NRRL 50797.

Refer to Table 1 in Appendix A for a summary of the product analysis data and terms of registration. Refer to Table 2 in Appendix A for a summary of the physical and chemical properties data.

B. Human Health Risk Assessment (40 CFR 158.2140)

1. Toxicity

The EPA has determined that all applicable mammalian toxicology data requirements for *Beauveria bassiana* strain ANT-03 are fulfilled. Acceptable Tier I mammalian toxicology data and information support the registration of the pesticide products and the request for an exemption from the requirement of a tolerance for residues of *Beauveria bassiana* strain ANT-03 in or on all food commodities. Tier II and Tier III studies were not required because of the lack of acute toxicity/pathogenicity in the Tier I studies.

The toxicity and pathogenicity tests and irritation tests (acute eye and primary dermal irritation) that address potential routes of exposure to the active ingredient are all classified in Toxicity Category III or IV and revealed little to no toxicity attributed to *Beauveria bassiana* strain ANT-03.

The overall conclusions from all toxicological data and information submitted by the applicant are briefly described below, in sections IV(B)(1)(a) and (b) and summarized in Table 3 in Appendix A.

a. Acute Toxicity/Pathogenicity – Tier I

Acute Oral Toxicity and Pathogenicity – Rat (Harmonized Guideline 885.3050; MRID No. 48974203): In an acute oral toxicity and pathogenicity study, groups of ~8-week-old Sprague-Dawley rats (12/sex) were given a single oral dose of *Beauveria bassiana* strain ANT-03 (1.2 x 10^{11} conidia/g) in sterile phosphate buffer saline (PBS) at a dose of 1.3 x 10^9 CFU/animal. The animals were observed for up to 21 days with interim scheduled sacrifices on Days 3, 7, and 14. Five males and five females were treated with inactivated test substance as controls; two males and two females were untreated "shelf controls"; and five males and five females were untreated controls. There were no treatment-related deaths, clinical signs, necropsy findings, or changes in body weight or body weight gain. The test organism was variously recovered from the blood, brain, spleen, lungs, liver, kidneys, or cecum contents through Day 7, and cleared by Day 14. The test organism cleared from the mesenteric lymph nodes by Day 21. Based on dissemination from an oral dose to dispersed internal regions of test animals, this organism may be infectious by oral exposure, though not pathogenic or toxic and is unlikely to grow at body temperatures.

EPA determined this study to be acceptable and concluded that *Beauveria bassiana* strain ANT-03 is not toxic or pathogenic orally at 1.3 x 10^9 CFU/animal.

Acute Pulmonary Toxicity and Pathogenicity – Rat (Harmonized Guideline 885.3150; MRID No. 48974204): In an acute pulmonary toxicity and pathogenicity study, groups of ~7 to 8-week-old Sprague-Dawley rats (15/sex) were exposed by the intratracheal route to *Beauveria bassiana* strain ANT-03 (1.2 x 10^{11} conidia/g) in sterile PBS at a dose of 1.1 x 10^9 CFU/animal. The animals were observed up to 21 days with interim scheduled sacrifices on Days 3, 7, and 14. Five males and five females were treated with autoclaved test substance as inactivated controls; four animals/sex were untreated "shelf controls"; and five animals/sex were untreated controls. Based on the results of this study, *Beauveria bassiana* strain ANT-03 was not toxic, infective, and/or pathogenic to rats when dosed at 1.1 x 10^9 CFU/animal. There were no test substance-related deaths, clinical signs, or necropsy findings. No test organisms were recovered from the blood, liver, kidneys, mesenteric lymph nodes, brain, and spleen or cecum contents at any time. Except for the Day 0 sacrifice, the test substance was not seen in the lungs at any time.

EPA determined this study to be acceptable and concluded that *Beauveria bassiana* strain ANT-03 is not toxic, infective, or pathogenic when inhaled at 1.1 x 10^9 CFU/animal.

Acute Intraperitoneal Injection Toxicity and Pathogenicity – Rat (Harmonized Guideline 885.3200; MRID No. 48974205): In an acute intraperitoneal injection toxicity and pathogenicity study, fifteen male and fifteen female albino Sprague-Dawley rats were injected with *Beauveria bassiana* strain ANT-03 (1.2 x 10^{11} conidia/g) in sterile PBS at a dose of 1.4 x 10^9 CFU/animal. The animals were treated on Day 0 and observed for up to 21 days. Three rats/sex treated with the test material were sacrificed one hour after dosing and the peritoneal cavity washed with PBS for dose verification and enumeration. The remaining animals were sacrificed and necropsied on Day 21. Two additional groups of five rats/sex served as untreated and inactivated MPCA controls and were also

sacrificed on Day 21. Based on the results of this study, *Beauveria bassiana* strain ANT-03 does not appear to be toxic when administered in a single dose to rats by IP injection at 1.4 x 10^9 CFU/animal. There were no treatment-related deaths, clinical signs, gross necropsy findings, or changes in body weight or body weight gain. Mesenteric lymph node weights in treated females were significantly lighter than untreated controls.

EPA determined this study to be acceptable and concluded that *Beauveria bassiana* strain ANT-03 is not toxic, infective, or pathogenic intraperitoneally at 1.4 x 10^9 CFU/animal.

Acute Dermal Toxicity – Rat (Harmonized Guideline 870.1200; MRID No. 48974206): In an acute dermal toxicity study, groups of five male and five female young adult Sprague-Dawley rats were dermally exposed to *Beauveria bassiana* strain ANT-03 (1.2 x 10^{11} conidia/g) moistened with deionized water (1.0 mL/g), applied to clipped application sites of approximately 10% of the body surface area for 24 hours at a dose level of 5,050 mg/kg body weight. The animals were treated on Day 0 and observed for a period of 14 days. There were no deaths or abnormal gross necropsy findings. All of the animals gained weight during both weeks of the study. There were no abnormal systemic clinical signs, and signs of dermal irritation were not detected at any time during the study.

EPA determined this study to be acceptable and concluded that *Beauveria bassiana* strain ANT-03 has a dermal NOAEL combined of > 5,050 mg/kg bw (EPA Toxicity Category IV).

Acute Oral Toxicity – Rat (Harmonized Guideline 870.1100)
Acute Dermal Toxicity – Rat (Harmonized Guideline 870.1200)
Acute Inhalation Toxicity – Rat (Harmonized Guideline 870.1300)
Primary Eye Irritation – Rabbit (Harmonized Guideline 870.2400)
Primary Dermal Irritation – Rabbit (Harmonized Guideline 870.2500)

In lieu of toxicology testing of *Beauveria bassiana* strain ANT-03, the applicant provided acceptable scientific justifications/rationales (MRID Nos. 48974213 [TGAI] and 48974302 [Bioceres EP]) to address the above listed data requirements, summarized below. The EPA generally agrees with the rationales provided, and the EPA determined the rationales to be acceptable to address the data requirements. (U.S. EPA, 2014a).

Oral gavage administration of the test material did not result in mortality, abnormal clinical signs, or gross pathology in rats at a single dose of 1.3 x 10^9 CFU/animal in an acute oral toxicity/pathogenicity study. As calculated by the reviewer, the administered dose of 1.3 x 10^9 CFU/rat equated to dose levels of approximately 36.7 to 65.7 mg/kg bw. In an acute pulmonary infectivity and toxicity study intratracheal administration of the test material (in phosphate buffered saline and Tween 20) at a single dose of 1.1 x 10^9 CFU/animal did not result in mortality, abnormal clinical signs, or gross pathology in rats. As calculated by the reviewer, the administered dose levels were approximately 31.4 to 56.2 mg/kg bw. TGAIs with *Beauveria bassiana* strains 447, GHA, and HF23 have been tested and classified in US EPA Toxicity Category III for primary eye irritation, and an EP formulated with *Beauveria bassiana* strain ATCC 74040 has also been classified in US EPA Toxicity Category III for primary eye irritation. In an acute dermal toxicity study, no skin irritation was seen when rats were dermally exposed to the test material moistened with deionized water (1.0 mL/g), applied to clipped application sites of approximately 10% of the body surface area for 24 hours at a dose level of 5,050 mg/kg bw.

There are no inert ingredients added to the TGAI. The inert ingredients added to the EP are present in food, recognized as safe, have tolerance exemptions, and are not expected to produce any toxic effects.

b. Acute Toxicology and Subchronic Toxicity/Pathogenicity – Tier II; Reproductive Fertility Effects, Carcinogenicity, Immunotoxicity, and Infectivity/Pathogenicity Analysis – Tier III

Tier II and Tier III studies were not required for *Beauveria bassiana* strain ANT-03, based on the lack of acute toxicity/pathogenicity in the Tier I studies.

c. Endocrine Disruptors

As required by the Administrator under the Federal Food, Drug, and Cosmetic Act (FFDCA) section 408(p), the EPA has developed the Endocrine Disruptor Screening Program (EDSP) and has begun to implement the screening program that is to be used to test all pesticides to determine whether certain substances (including pesticide active and other ingredients) may have an effect in humans or wildlife similar to an effect produced by a "naturally occurring estrogen, or other such endocrine effects as the Administrator may designate." FFDCA section 408(p)(4), authorizes the Administrator, by order, to exempt from the requirements of the Endocrine Disruptor Screening Program a biologic substance or other substance if a determination is made that the substance is anticipated not to produce any effect in humans similar to an effect produced by a naturally occurring estrogenic substance. Between October 2009 and February 2010, EPA issued test orders/data call-ins for the first group of 67 chemicals, which contain 58 pesticide active ingredients and nine inert ingredients. *Beauveria bassiana* strain ANT-03 is not among the group of 58 pesticide active ingredients on the initial list to be screened under the EDSP.

The Agency believes that *Beauveria bassiana* strain ANT-03 likely is a substance that would not produce any effect in humans similar to an effect produced by a naturally occurring estrogenic substance. As such, and pursuant to Section 408(p)(4), EPA will determine in the future whether it can exempt *Beauveria bassiana* strain ANT-03 from the requirements of the Section 408(p) EDSP. In the event the Agency does determine to exempt this substance from the EDSP, an order will be issued. For further information on the status of the EDSP, the policies and procedures, the list of 67 chemicals, future lists, the test guidelines and the Tier 1 screening battery, please visit our website: *http://www.epa.gov/endo/*.

2. Federal Food, Drug, and Cosmetic Act (FFDCA) Considerations

Section 408(c)(2)(A)(i) of FFDCA allows the EPA to establish an exemption from the requirement for a tolerance (the legal limit for a pesticide chemical residue in or on a food) only if the EPA determines that the exemption is "safe." Section 408(c)(2)(A)(ii) of FFDCA defines "safe" to mean that "there is a reasonable certainty that no harm will result from aggregate exposure to the pesticide chemical residue, including all anticipated dietary exposures and all other exposures for which there is reliable information." This includes exposure through drinking water and in residential settings, but it does not include occupational exposure. Pursuant to

section 408(c)(2)(B) of FFDCA, in establishing or maintaining in effect an exemption from the requirement of a tolerance, the EPA must take into account the factors set forth in section 408(b)(2)(C) of FFDCA, which require the EPA to give special consideration to exposure of infants and children to the pesticide chemical residue in establishing a tolerance exemption, and to "ensure that there is a reasonable certainty that no harm will result to infants and children from aggregate exposure to the pesticide chemical residue...." Additionally, section 408(b)(2)(D) of FFDCA requires that the EPA consider "available information concerning the cumulative effects of [a particular pesticide's] . . . residues and other substances that have a common mechanism of toxicity."

The EPA performs a number of analyses to determine the risks from aggregate exposure to pesticide residues. First, the Agency determines the toxicity of a pesticide. Second, the EPA examines exposure to the pesticide through food, drinking water, and through other exposures that occur as a result of pesticide use in residential settings. Consistent with section 408(b)(2)(D) of FFDCA, the EPA has reviewed the available scientific data and other relevant information, and considered its validity, completeness, and reliability and the relationship of this information to human risk. The Agency also considered available information concerning the variability of the sensitivities of major identifiable subgroups of consumers, including infants and children. Based on the acute toxicity/pathogenicity data and information discussed previously and presented in Table 3 in Appendix A, the data required for a FFDCA risk assessment for *Beauveria bassiana* strain ANT-03 have been fulfilled.

a. Aggregate Exposure

In examining aggregate exposure, section 408 of FFDCA directs EPA to consider available information concerning exposures from the pesticide residue in food and all other nonoccupational exposures, including drinking water from ground water or surface water and exposure through pesticide use in gardens, lawns, or buildings (residential and other indoor uses). Due to the biological nature of *Beauveria bassiana* strain ANT-03, it is not expected to persist in the environment or accumulate in any reservoirs; rather it will naturally degrade due to consumption by other biological organisms and exposure to heat, cold, sunlight, etc.

Food Exposure and Risk Characterization: *Beauveria bassiana* strains are widely found in soils and insects worldwide. The EPA found that increased dietary exposure to *Beauveria bassiana* strain ANT-03, a naturally occurring bacterium pathogenic to insects and commonly found in agricultural settings and on fresh produce, is anticipated to be negligible. Further, *Beauveria bassiana* is not known to produce any mammalian toxins, and no foodborne disease outbreaks associated with *Beauveria bassiana* or toxin production from *Beauveria bassiana* have been reported. The EPA concluded that the risk posed to adults, infants, and children is likely to be minimal because of the low acute oral toxicity/pathogenicity potential of *Beauveria bassiana* strain ANT-03.

Drinking Water Exposure and Risk Characterization: Since *Beauveria bassiana* is naturally present in soils and insects, exposure to *Beauveria bassiana* from surface water and possibly groundwater can be expected. Exposure to residues of *Beauveria bassiana* strain ANT-03 in consumed drinking water may occur as a result of spray drift from aerial and ground applications

or runoff of prepared fields and rangelands into surface waters. However, if *Beauveria bassiana* strain ANT-03 were to be transferred to surface water or groundwater intended for eventual human consumption (e.g., through spray drift or runoff) and directed to wastewater treatment systems or drinking water facilities, water treatment processes would remove any *Beauveria bassiana* present. It likely would not survive the conditions water is subjected to in such systems or facilities, including chlorination, pH adjustments, filtration, and/or occasionally high temperatures (Centers for Disease Control and Prevention, 2015; U.S. EPA, 2004). In the remote likelihood that *Beauveria bassiana* strain ANT-03 is present in drinking water (e.g., water not subject to treatment systems or facilities), its target pest specificity and available toxicity and pathogenicity data indicate no toxicity and/or pathogenicity is likely to occur with any drinking water exposure to *Beauveria bassiana* strain ANT-03 that results from pesticide applications made in accordance with good agricultural practices (see section IV(B)(1)(a) and Table 3 in Appendix A).

Non-occupational, Residential Risk Characterization: Given the home and garden proposed use pattern for *Beauveria bassiana* strain ANT-03, non-occupational exposure is expected. However, repeated exposures to *Beauveria bassiana* strain ANT-03 from pesticidal applications will not exceed he EPA's level of concern in light of the available data that demonstrate *Beauveria bassiana* strain ANT-03 is not toxic (acute dermal toxicity and acute pulmonary toxicity/pathogenicity), is non-irritating (primary dermal irritation), and is not pathogenic (acute pulmonary toxicity/pathogenicity and acute injection toxicity/pathogenicity) when used as labeled in accordance with good agricultural practices (see section IV(B)(1)(a) and Table 3 in Appendix A).

b. Cumulative Effects from Substances with a Common Mechanism of Toxicity

Section 408(b)(2)(D)(v) of FFDCA requires that, when considering whether to establish, modify, or revoke a tolerance exemption, the EPA consider "available information concerning the cumulative effects of [a particular pesticide's] . . . residues and other substances that have a common mechanism of toxicity."

Beauveria bassiana strain ANT-03 is not toxic via the dietary, dermal and pulmonary routes of exposure. For the purposes of the tolerance action, therefore, the EPA has concluded that *Beauveria bassiana* strain ANT-03 does not have a common mechanism of toxicity with other substances. Thus, section 408(b)(2)(D)(v) of the FFDCA does not apply.

c. Determination of Safety for U.S. Population, Infants and Children

In considering the establishment of a tolerance or tolerance exemption for a pesticide chemical residue, FFDCA section 408 (b)(2)(C) provides that the EPA shall assess the available information about consumption patterns among infants and children, special susceptibility of infants and children to pesticide chemical residues, and the cumulative effects on infants and children of the residues and other substances with a common mechanism of toxicity. In addition, FFDCA section 408 (b)(2)(C) provides that the EPA shall apply an additional tenfold (10X) margin of exposure (safety) for infants and children in the case of threshold effects to account for prenatal and postnatal toxicity and the completeness of the database on toxicity and exposure unless the EPA determines that a different margin of exposure (safety) will be safe for infants

and children. This additional margin of exposure (safety) is commonly referred to as the Food Quality Protection Act Safety Factor. In applying this provision, the EPA either retains the default value of 10X or uses a different additional safety factor when reliable data available to the EPA support the choice of a different factor.

Based on the acute toxicity and pathogenicity data/information discussed in section IV(B)(1)(a) and Table 3 in Appendix A, the EPA concludes that there are no threshold effects of concern to infants, children, or adults when *Beauveria bassiana* strain ANT-03 is used as labeled in accordance with good agricultural practices. As a result, the EPA concludes that no additional margin of exposure (safety) is necessary to protect infants and children and that not adding any additional margin of exposure (safety) will be safe for infants and children.

Moreover, based on the same data/information and the EPA's analysis as presented directly above, the Agency is able to conclude that there is a reasonable certainty that no harm will result to the United States population, including infants and children, from aggregate exposure to the residues of *Beauveria bassiana* strain ANT-03 when it is used—as labeled and in accordance with good agricultural practices—as an insecticide. Such exposure includes all anticipated dietary exposures and all other exposures for which there is reliable information. The EPA has arrived at this conclusion because, considered collectively, the data and information available on *Beauveria bassiana* strain ANT-03 do not demonstrate toxic or pathogenic potential to mammals, including infants and children. No toxicological, irritation or pathogenic endpoints were noted in mammalian testing of *Beauveria bassiana* strain ANT-03 and differential effects to infants and children are not expected.

3. Occupational Exposure and Risk Characterization

The available data (e.g., lack of toxicity noted for oral, dermal, and inhalation routes of exposure to *Beauveria bassiana* strain ANT-03) do not demonstrate toxic potential to persons occupationally exposed to this microbial pest control agent. Standard personal protective equipment (PPE) on the label (mixer and handler use of an N-95 equivalent respirator) further mitigates unintentional exposures.

4. Human Health Risk Characterization

The EPA considered human exposure to *Beauveria bassiana* strain ANT-03 in light of the standard for registration in FIFRA. A determination has been made that no unreasonable adverse effects to the U.S. population in general, and to infants and children in particular, will result when the *Beauveria bassiana* strain ANT-03 manufacturing-use pesticide product is used to formulate end-use pesticide products in accordance with EPA-accepted labeling.

C. Environmental Assessment (40 CFR § 158.2150)

Guideline studies and waiver rationale were submitted to meet data requirements for non-target organisms per 40 CFR § 158.2150 for *Beauveria bassiana* strain ANT-03. These data and waiver rationale are sufficient to fulfill the relevant microbial pesticide data requirements and for risk assessment purposes. Further testing of non-target organisms at higher tier levels (i.e., Tiers II,

III, and IV) is not required. The EPA's scientists made a "No Effect" determination for federally listed threatened and endangered mammals, birds, and terrestrial and aquatic plants. Effects to listed freshwater and marine/estuarine fish and invertebrates and insects could not be precluded, and a determination requires additional analysis. Adverse effects to all other non-target organisms are not expected to result from the registration of *Beauveria bassiana* strain ANT-03 when applied in accordance with the directions on the label.

For a summary of the generic data requirements described in sections IV(C)(1), refer to Table 4 in Appendix A.

1. Ecological Exposure and Risk Characterization

According to the label for the EP, *Beauveria bassiana* strain ANT-03 is intended for use on agricultural crops, on turf, in ornamental and landscape use sites, and home gardens for the control or suppression of many foliar-feeding pests, including aphids, white flies, thrips, plant bugs, beetles and weevils infesting labeled crops or use sites. The end-use product, Bioceres, containing *Beauveria bassiana* strain ANT-03 is a wettable powder (Bioceres-WP) or water-dispersible briquette (Bioceres-WB) that is to be mixed in water and applied as a foliar spray with ground or aerial equipment fit for conventional insecticide spraying or by chemigation, in field or greenhouse use sites at a rate of up to 6 lbs/acre (2.72×10^{13} CFU/acre). When mixed in the minimum amount of water suggested on the label (50 gallons), the concentration of the EP that is applied to the environment is 0.014 g/mL, resulting in a concentration of the a.i. of 3.8×10^{7} CFU/mL.

The label states that for optimal results, proper timing of the application of *Beauveria bassiana* ANT-03 targeting newly hatched larvae is important. The label also states that thorough coverage of infested plant parts is necessary for effective control, and applications can be repeated at 5- to 7-day intervals. Single applications to terrestrial environments can lead to exposures to non-target organisms in both terrestrial and aquatic environments. Repeated applications at the higher label rates may lead to elevated concentrations, which in turn can result in a greater potential for non-target exposure in terrestrial and/or nearby aquatic environments. Spray drift from foliar applications may result in some exposure to nearby areas, including aquatic environments. While *Beauveria bassiana* ANT-03 is a new strain isolated originally from a dead insect in Canada, *Beauveria bassiana* is known to occur naturally in soil and water in various regions of the world (Zimmerman, 2007), so soil- and water-borne non-target organisms in those regions currently have some exposure to the existing fungal strains.

a. Terrestrial Non-Target Animals

Birds and Mammals
Avian oral toxicity/pathogenicity – Harmonized Guideline 885.4050; MRID No. 48974207
Avian inhalation toxicity – Harmonized Guideline 885.4100; MRID No. 48974213
Wild mammal toxicity/pathogenicity – Harmonized Guideline 885.4150; MRID Nos. 48974203, 48974204, and 48974205

The guideline study submitted for the avian oral toxicity/pathogenicity testing requirement

showed no adverse effects in birds tested (Northern bobwhite, *Colinus virginianus*). However, the study is classified as supplemental because the birds were not tested at the maximum hazard dose. Nonetheless, *Beauveria bassiana* strain ANT-03 is not likely to grow at a bird's high body temperatures (see McNab (1966)) and adverse effects to birds exposed via the oral route are considered to be unlikely.

Based on expected lack of adverse effects observed in birds exposed to *Beauveria bassiana* strain ANT-03, risk to non-target birds resulting from the registration is not likely significant.

An acute oral toxicity/pathogenicity study with laboratory rats indicated no adverse effects of *Beauveria bassiana* strain ANT-03 when administered at a dose of 1.3 X 10^9 CFU/animal (MRID No. 48974203). An acute pulmonary toxicity/pathogenicity study was also submitted (MRID No. 48974204) that indicated *Beauveria bassiana* was not toxic, infective, or pathogenic to rats at a dose of 1.1 x 10^9 CFU/animal. An acute intraperitoneal (IP) injection toxicity and pathogenicity study (MRID No. 48974205) does not appear to show toxicity when administered in a single dose to rats by IP injection at 1.4 x 10^9 CFU/animal (U.S. EPA, 2014a).

Some wild mammals may be exposed to *Beauveria bassiana* strain ANT-03 in treated areas. Since adverse effects were not observed in the mammalian studies, risk to wild mammals is expected to be low from the registration of the new active ingredient.

Non-target Insect and Honey Bee Testing
Non-target insect testing – Harmonized Guideline 885.4340; MRID Nos. 48974210, 48974211, and 48974213
Honey bee testing – Harmonized Guideline 885.4380; MRID No. 48974213

The guideline study submitted to meet the requirement for non-target insect testing showed no toxicity to ladybird beetles (*Hippodamia convergens*) upon a 10-day dietary exposure to corn earworm (*Helicoverpa zea*) sprayed with *Beauveria bassiana* ANT-03 at 1 x 10^6 CFU/mL. However, reduced food consumption was observed with the test substance group during Days 7-11 when compared to the untreated control group. It is not possible to determine potential toxicity or pathogenicity in this study, since reduced food consumption could be the result of toxicity/pathogenicity, or could have resulted in reduced exposure during the study. Therefore, these results are inconclusive.

The results with green lacewing larvae indicated no significant differences in mortality among different test groups when exposed to *Beauveria bassiana* ANT-03 at 1 x 10^6 CFU/mL in a 25-day dietary toxicity/pathogenicity study. However, the mean number of eggs consumed per day during Days 0-7 in the untreated control group, inactivated test substance group, and test substance group was 19.3, 14.5, and 16.3, respectively. The inactivated test substance group and the test substance group consumed significantly fewer eggs during Days 0-7 than did the untreated control group ($p<0.0001$). (The presence or absence of the surfactant in the various test groups was not clear. Upon request for clarification, the applicant submitted additional information that stated that only test substance and inactive test substance groups included the surfactant (MRID No. 49453101) but not the untreated control. Surfactants can cause adverse effects in some insects.) The reduced egg consumption observed might be due to the surfactant,

but this cannot be confirmed without having a control group treated with the surfactant alone. Without this control, results are inconclusive.

The third guideline insect study was not submitted, but a data waiver was requested. The rationale to support the request for no additional testing was not sufficient to conclude that adverse effects to non-target insects is unlikely.

In light of the observation of reduced food consumption in adult ladybird beetles tested and the fact that *Beauveria bassiana* is an entomopathogen known to have a wide host range, the EPA cannot conclude that adverse effects to non-target insects will not occur. It should be best to exercise caution with the uses of the new active ingredient: e.g., to avoid the use of the new active ingredient on or around the crops that are pollinated by insects. The language in the Use Directions on the label under the heading "SENSITIVE AREAS" states, "[t]he pesticide should only be applied when the potential for drift to adjacent sensitive areas (e.g., residential areas, bodies of water, known habitat for threatened or endangered species, non-target crops) is minimal (e.g., when wind is blowing away from the sensitive areas)," which may serve to reduce exposure. Additional language will be required to alert users to the potential for adverse effects to insects, including beneficial species.

A study with honey bees was not provided to support the honey bee data requirement for the proposed FIFRA section 3 registration of *Beauveria bassiana* strain ANT-03. A data waiver was requested with rationale that included a reference (Al-mazra'awi, 2006) that involved honey bees as a vector for the dispersal of another strain of *Beauveria bassiana* for control of tarnished plant bug on canola, without observable impact on honey bees. Given that *Beauveria bassiana* species is a known insect pathogen and honey bee is a major pollinating insect that plays an important role agriculturally as well as environmentally, the absence of the required Tier 1 Honey Bee study is likely to increase concerns about the effects of *Beauveria bassiana* ANT-03 on honey bees. In addition, the existing data may not be sufficient to predict the effects of *Beauveria bassiana* strain ANT-03, a unique strain, on other beneficial non-target insects. A vector technology has been developed to use honey bees to disseminate conidia of *Beauveria bassiana* to control target pests such as whiteflies and thrips (Shipp, 2009), but studies like this generally are not intended to study the direct effects of *Beauveria bassiana* on honey bees or the bee hives, but to evaluate the effectiveness of honey bee as a vector to transfer the microbial pest control agent. One study (Al Mazra'awi et al., 2007) did examine the effects of another strain of *Beauveria bassiana* on honey bees. In this study, mortality was low and not significantly different from that of the controls; however, mycosis in bee cadavers from *Beauveria bassiana* treated hives indicated the potential for *Beauveria bassiana* to cause mortality in honey bees.

Based on the above information, the rationale provided by the applicant is not sufficient to determine conclusively that *Beauveria bassiana* strain ANT-03 will not adversely affect honey bees or that applications to insect pollinated crops will not cause bee mortality. Thus, a statement is required to be added to caution users about the potential effects on bees and other pollinators, and specifically restrict users from applying the end-use product when bees are visiting crops and surrounding areas. This language is likely to reduce exposure, but may not eliminate it completely.

b. Aquatic Non-Target Animals

Freshwater Fish and Invertebrate Testing
Freshwater Fish Toxicity/Pathogenicity – Harmonized Guideline 885.4200; MRID No. 48974208
Freshwater Invertebrate Toxicity/Pathogenicity – Harmonized Guideline 885.4240; MRID No. 48974209

A guideline study submitted for the non-target freshwater fish testing requirements indicated no toxicity/pathogenicity of *Beauveria bassiana* ANT-03 to *Oncorhynchus mykiss* (rainbow trout) when tested at 1 x 10^6 CFU/mL. However, one of the three replicates of the test substance group (10 of the 30 fish in that group) was not included in the mortality calculations. The study author's explanation for the mortality was that this was likely due to a temporary lack of aeration/drop in dissolved oxygen in the test containers, but no other details (e.g., analysis of the dead fish for the MPCA and/or necropsy) were provided.

A guideline study for the non-target freshwater invertebrate testing showed toxicity to *Daphnia magna* at 1 x 10^6 CFU/mL. Mortality reached 100% in the 1 x 10^6 test substance group (*Beauveria bassiana* strain ANT-03) by Day 8 and 20% in the 1 x 10^3 test substance group in 21 days. The study is classified as supplemental for the following reasons: The study indicates that *Beauveria bassiana* strain ANT-03 is toxic to *Daphnia* when tested at 1 x 10^6 conidia/mL. The study describes the EC_{50} values for survival, reproduction and biomass. However, the significance of these EC_{50} values are uncertain, because the study was not designed properly to calculate a definitive EC_{50}. In the daphnia study, two concentrations were tested at 10^3 and 10^6 CFU/mL. Since 100% mortality was observed at the high rate and 20% mortality was observed at the low rate, it can be concluded that the LC_{50} is >10^3 CFU/mL. More testing at concentrations lower than 10^6 CFU/mL but higher than 10^3 CFU/mL is needed to support the EC_{50} values presented in the *Daphnia* study.

The EPA Standard Wetland, which is a wetland of 1 acre in size and 15 cm in depth, can be used to determine a worst-case scenario estimate for estimated environmental concentration (EEC) in water. The EEC for water is calculated to be 4.49 x 10^5 CFU/mL ([6 lbs (product/acre) X (454 g/lb) X (1 X 10^{10} CFU/g product) X (1 acre/40,468,564 cm^2)] ÷ 15 cm. Using the EPA Standard Pond, which is a pond of 1 acre in size and 6 ft (182.88 cm) deep, the EEC would be 3.7 x10^3 CFU/mL. To gain a more realistic EEC based on the potential for spray drift, the Tier I aerial agricultural application scenario in the AgDRIFT spray drift model can be applied to these numbers. Under that scenario assuming fine to medium droplet size, a maximum of 12.5% of the amount applied is expected to reach aquatic areas at the water's edge. Therefore, the EEC for the wetland and pond can be refined to 5.6 x 10^4 CFU/mL and 4.62 x 10^2 CFU/mL. Since the potential for exposure falls above 10^3 CFU/mL from the *Daphnia* study described above, adverse effects to aquatic invertebrates in some aquatic systems cannot be ruled out, especially since repeated applications may increase exposure.

To address this, the EPA will be requiring a spray drift buffer of 50 ft from the water's edge. This buffer will reduce exposure to 3.1 x 10^4 CFU/mL in the standard wetland and 2.59 x 10^2 CFU/mL in the standard pond. While it cannot be assured that such a spray buffer would

eliminate the potential for adverse effects in all cases, the EPA feels confident that this buffer would provide sufficient protection for aquatic organisms to address any risk concerns.

Estuarine/Marine Fish and Invertebrate Testing
Estuarine/Marine Fish and Invertebrate Testing – Harmonized Guideline 885.4280; MRID No. 48974213

The applicant did not submit a guideline study to meet the requirement for non-target marine/estuarine fish and invertebrate testing, but submitted rationale in lieu of testing. This testing is conditionally required if significant exposure of non-target marine/estuarine fish and invertebrates to *Beauveria bassiana* strain ANT-03 is expected. The rationale submitted by the applicant is supplemental as it relied mainly on the freshwater fish and invertebrate toxicity/pathogenicity testing results. The EPA generally does not rely on results of testing with freshwater species to support risk conclusions on marine/estuarine species. Also, as described above, the rainbow trout study results were inconclusive, and the *Daphnia* study indicated adverse effects were possible, though at an unknown level of exposure.

The condition for requiring estuarine/marine fish and invertebrate studies would depend on whether there would be significant exposure of aquatic organisms to *Beauveria bassiana* ANT-03, which in turn would depend on both the type/amount of application (singular or multiple; foliar or soil surface) and the proximity of the treated areas to aquatic environments.

Given the potential toxicity of *Beauveria bassiana* ANT-03 to rainbow trout and *Daphnia* and also potential exposure, as described above for freshwater environments, especially when applied repeatedly, adverse effects on other non-target aquatic organisms (both in freshwater and in marine/estuarine waters) are expected to occur.

To address this risk, EPA will be requiring a spray drift buffer of 50 ft from the water's edge, as described above. While it cannot be assured that such a spray buffer would eliminate the potential for adverse effects in all cases, the EPA feels confident that this buffer would provide sufficient protection for aquatic organisms to address any risk concerns.

c. Non-Target Plants

Terrestrial and Aquatic Plants
Non-target Plant Testing – Harmonized Guideline 885.4300; MRID No. 48974213

A guideline study for Non-target Plant Testing, Tier 1 was not submitted, but a data waiver was requested. Scientific rationale submitted for a waiver of the requirement for Non-target Plant Testing consists of the following: *Beauveria bassiana* ANT-03 is a naturally occurring soil fungus whose level in the environment would not significantly increase with the registration of the new active ingredient, and the uses of the new a.i. on agricultural crop, turf, ornamental and landscape, and home and garden use sites is not expected to result in increased exposure to non-target plants.

Beauveria bassiana ANT-03 is a newly isolated strain from a dead insect in Canada, with new

uses (e.g., for terrestrial and food). This strain was stated to be not phytotoxic to certain crops in research and development, but data were not provided to support the claim of no phytotoxicity. Scientific rationale based solely on the published information on existing strains is not sufficient for the waiver request for the new a.i. However, while *Beauveria bassiana* is a known entomopathogen, it is generally not known as a plant pathogen. Therefore, the proposed uses of *Beauveria bassiana* ANT-03 do not exceed the EPA's level of concern.

2. Environmental Fate Assessment Data

As the data and information provided are sufficient to fulfill the Tier I non-target organism data requirements and mitigation language will address any identified non-target organism risks and uncertainties associated with *Beauveria bassiana* ANT-03, further testing at higher levels (Tiers II, III, and IV) is not required.

3. Threatened and Endangered Species Assessment

The EPA has determined that no adverse effects are anticipated for most non-target species exposed to *Beauveria bassiana* strain ANT-03 as a result of the labeled applications, including mammals, birds, terrestrial and aquatic plants. Therefore, a "No Effect" determination is made for direct and indirect effects to federally listed endangered and threatened ("listed") mammals, birds, terrestrial and aquatic plants and for their designated critical habitat.

Adverse effects to freshwater and marine/estuarine fish and invertebrates and non-target insects cannot be ruled out. Therefore, direct effects to individuals of listed insect species cannot be precluded, nor can indirect effects to listed species that depend on these organisms or effects on Critical Habitat related to these organisms. Additional analysis of the potential effects is necessary to make a determination for these species.

V. ENVIRONMENTAL JUSTICE

EPA seeks to achieve environmental justice—the fair treatment and meaningful involvement of all people regardless of race, color, national origin, or income—with respect to the development, implementation, and enforcement of environmental laws, regulations, and policies. Fair treatment means that no group of people, including racial, ethnic, or socioeconomic groups, should bear a disproportionate share of the negative environmental consequences resulting from industrial, municipal, and commercial operations or the execution of federal, state, local, and tribal environmental programs and policies. Meaningful involvement means that (1) potentially affected community residents have an appropriate opportunity to participate in decisions about a proposed activity that will affect their environment and/or health; (2) the public's contribution can influence the regulatory agency's decision; (3) the concerns of all participants involved will be considered in the decision-making process; and (4) the decision-makers seek out and facilitate the involvement of those potentially affected. EPA has this goal for all communities and persons across the U.S.

To help address potential environmental justice issues, during the 15-day public participation comment period, the EPA sought information on any groups or segments of the population who,

as a result their location, cultural practices, or other factors, may have atypical, unusually high exposure to *Beauveria bassiana* strain ANT-03, compared to the general population. The EPA received no public comments on this particular matter.

For additional information regarding environmental justice issues, please visit EPA's web site at *http://www.epa.gov/compliance/environmentaljustice/index.html*.

VI. RISK MANAGEMENT DECISION

Section 3(c)(5) of FIFRA provides for the registration of a pesticide provided that all the following determinations are made:

(1) its composition is such as to warrant the claims for it;
(2) its labeling and other materials required to be submitted comply with the requirements of FIFRA;
(3) it will perform its intended function without unreasonable adverse effects on the environment; and
(4) when used in accordance with widespread and commonly recognized practice, it will not generally cause unreasonable adverse effects on the environment.

To satisfy criterion one (1), the *Beauveria bassiana* strain ANT-03 pesticide products have well-known properties. The EPA has no knowledge that would contradict the claims made on the label for the manufacturing-use pesticide product, *Beauveria bassiana* strain ANT-03 Technical, and the end-use pesticide product is not expected to cause unreasonable adverse effects on the environment when used according to the label instructions. Criterion two (2) is satisfied by the current product labels, as well as the data and information presented in this document. The EPA believes that the *Beauveria bassiana* strain ANT-03, will not cause any unreasonable adverse effects on the environment, and the end-use product is likely to control pests, satisfying criterion three (3). Criterion four (4) is satisfied in that the *Beauveria bassiana* strain ANT-03 is not expected to cause unreasonable adverse effects when used according to label instructions. Therefore, *Beauveria bassiana* strain ANT-03 Technical and Bioceres, the end-use pesticide product, are eligible for registration under FIFRA section 3(c)(5) for the labeled uses.

VII. ACTIONS REQUIRED OF THE REGISTRANT

A. Final Printed Labeling

Before releasing the pesticide products containing *Beauveria bassiana* strain ANT-03 for shipment, the registrant is required to provide appropriate final printed labeling to the EPA.

B. Terms of Registration

As terms of the registration, the applicant must provide the following:

1) a manufacturing process amended to describe a method for identifying spores as *Beauveria bassiana* strain ANT-03;
2) batch analyses using viable count calculations;
3) specific methods for microbial contamination analyses;
4) defined rejection limits for contaminants; and
5) an explanation of unidentified fungi.

Data and information to address these five terms are required not due to risk concerns, but to clarify and define the quality assurance/quality control measures. Receipt by the EPA is required within 90 days of the technical product's registration issuance date, except that 12 months are allowed for the submission addressing the batch analyses requirement (term two).

The end-use product label must include the following hazard statement under the Environmental Hazards section to address ecological risk concerns and uncertainties:

> *"This product may harm beneficial insects and honey bees. Do not apply around bee hives. Do not apply while bees and other pollinating insects are actively foraging. This product may be harmful to aquatic organisms. Drift and runoff may be hazardous to aquatic organisms in water adjacent to treated areas. Do not apply within 50 feet of aquatic habitats (such as, but not limited to, lakes, reservoirs, rivers, streams, marshes, ponds, estuaries, and commercial fish ponds)."*

C. Reporting of Adverse Effects and Hypersensitivity Incidents

Notwithstanding the information stated in the previous sections, it should be clearly understood that certain specific data are required to be reported to the EPA as a requirement for maintaining the federal registration for a pesticide product. Reports of all incidents of adverse effects to the environment must be submitted to the EPA under the provisions stated in FIFRA section 6(a)(2). Additionally, all incidents of hypersensitivity (including both suspected and confirmed incidents) must be reported to the EPA under the provisions of 40 CFR § 158.2140(d).

VIII. BIBLIOGRAPHY

For definitions of scientific terms, please refer to *http://www.epa.gov/pesticides/glossary/*.

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48974201	Roberts, A. (2013) Product Chemistry for Beauveria bassiana Strain ANT-03 Technical: (Group B). Unpublished study prepared by Technology Sciences Group, Inc. 228p.	03-May-2013
48974202	Mayrand-Provencher, L. (2013) Analysis of Beauvericin in the Fungus (Species) Beauveria bassiana (Bals.) Vuill Strain: ANT-03 Stage: Aerial conidia. Project Number: 230/2013/01/OCR. Unpublished study prepared by Eliapharma Services, Inc. 32p.	03-May-2013
48974203	Monds, K. (2011) Bioterra (Beauveria bassiana): Acute Oral Toxicity/Pathogenicity Study in Rats: Final Report. Project Number: 14817/11/OCR. Unpublished study prepared by Stillmeadow, Inc. 31p.	03-May-2013
48974204	Monds, K. (2011) Bioterra (Beauveria bassiana): Acute Pulmonary Toxicity/Pathogenicity Study in Rats: Final Report. Project Number: 15020/11/OCR. Unpublished study prepared by Stillmeadow, Inc. 28p.	03-May-2013
48974205	Monds, K. (2011) Bioterra (Beauveria bassiana): Acute Intraperitoneal Injection Toxicity/Pathogenicity Study in Rats: Final Report. Project Number: 14818/11/OCR. Unpublished study prepared by Stillmeadow, Inc. 22p.	03-May-2013
48974206	Kuhn, J. (2011) Bioterra (Beauveria bassiana): Acute Dermal Toxicity Study in Rats: Final Report. Project Number: 15014/11/OCR. Unpublished study prepared by Stillmeadow, Inc. 12p.	03-May-2013
48974207	Younger, C. (2011) Bioterra (Beauveria bassiana): Acute MPCA Oral Toxicity Study in Bobwhite Quail: Final Report. Project Number: 14819/11/OCR. Unpublished study prepared by Stillmeadow, Inc. 18p.	03-May-2013
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48974210	Younger, C. (2011) Bioterra (Beauveria bassiana): Ladybird Beetle Non-Target Insect Microbial Testing: Final Report. Project Number: 15017/11/OCR. Unpublished study prepared by Stillmeadow, Inc. 14p.	03-May-2013
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48974212	Roberts, A. (2013) Summary of Data on Beauveria bassiana conidia Field Persistence. Unpublished study prepared by Anatis Bioprotection, Inc. 48p.	03-May-2013
48974213	Roberts, A. (2013) Response to Tier 1 Microbial Pesticide Data Requirements for Beauveria bassiana strain ANT-03 Technical. Unpublished study prepared by Technology Sciences Group, Inc. 136p.	03-May-2013
49453100	Anatis Bioprotection, Inc. (2014) Submission of Toxicity Data in Support of the Registration of Beauveria bassiana strain ANT-03 Technical. Transmittal of 1 Study.	22-Aug-2014
49453101	Roberts, A. (2014) Response to EPA Questions for Beauveria bassiana Strain ANT-03 Technical Testing. Project Number: 15017/11, 10018/11. Unpublished study prepared by Technology Science Group, Inc. 110p.	22-Aug-2014

B. Studies Submitted to Support the Registration of Pesticide End-Use Product Containing *Beauveria bassiana* strain ANT-03

MRID	Citation	Receipt Date
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48974301	Roberts, A. (2013) Product Chemistry for Bioceres. Unpublished study prepared by Technology Sciences Group, Inc. 69p.	03-May-2013

MRID	Citation	Receipt Date
48974302	Roberts, A. (2013) Response to Tier 1 Microbial Pesticide Data Requirements for Bioceres. Unpublished study prepared by Technology Sciences Group Inc. 41p.	03-May-2013

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APPENDIX A. MICROBIAL PESTICIDES DATA REQUIREMENTS (40 CFR PART 158 – SUBPART V)

TABLE 1. Product Analysis Data Requirements for the TGAI *Beauveria bassiana* strain ANT-03 and the End-Use Pesticide Product (EP), Bioceres (40 CFR § 158.2120)

Harmonized Guideline Number	Data Requirement	Results Summary	MRID No. (TGAI)	MRID No. (Bioceres)
885.1100	Product Identity	Submitted data fulfill the requirement for product identity, manufacturing process, and discussion of formation of unintentional ingredients. The EPA is requiring as a term of registration of the TGAI the following: - That a method for spore identification be added to the manufacturing process; - That specific methods for microbial contamination analyses be provided; - That rejection limits for contaminants be defined; and - That unidentified fungi be explained. *Beauveria bassiana* strain ANT-03 Technical contains 100.0% by weight *Beauveria bassiana* strain ANT-03, containing not less than 2.2 x 10^{10} CFU/g Bioceres is an end-use product containing 20% w/w *Beauveria bassiana* strain ANT-03 and not less than 1 x 10^{10} CFU ai/g.	48974201 48974202 49453101	48974301
885.1200	Manufacturing Process			
885.1300	Discussion of Formation of Unintentional Ingredients			
885.1400	Analysis of Samples	Submitted data fulfill the requirement. For the TGAI, the EPA is requiring submission of batch analyses using viable count calculations with the TGAI.		
885.1250	Deposition of a Sample in a Nationally Recognized Culture Collection	A sample of *Beauveria bassiana* strain ANT-03 is on deposit with the Agricultural Research Service Culture Collection (NRRL) under NRRL 50797		
885.1500	Certification of Limits	Limits are adequate/acceptable.		

TABLE 2. Physical and Chemical Characteristics for *Beauveria bassiana* strain ANT-03 and the End-Use Pesticide Product (EP), Bioceres (40 CFR § 158.2120)			
Guideline Reference No./Property		**Description of Results - *Beauveria bassiana* strain ANT-03 TGAI MRID No. 48974201**	**Description of Results – Bioceres MRID No. 48974301**
830.6302	Color	Cream-white	Not applicable, per 40 CFR § 158.2120(c)
830.6303	Physical State	Powder	
830.6304	Odor	Odorless	
830.6313	Stability	Stable refrigerated 1 year, stable 6 months at room temp. Expected with metals	
830.6317	Storage Stability	Stable under refrigerated conditions (4± 2º C) for 1 year	Stable 1 year 4+/-2 degrees C; 6 months 21 degrees C in vacuum-sealed bags; 3 months in non-vacuum-sealed bags.
830.6319	Miscibility	Not applicable, not an emulsifiable liquid or diluted with petroleum solvents	Not applicable, per 40 CFR § 158.2120(d)(2)
830.6320	Corrosion Characteristics	No observed corrosion or packaging degradation during 1-year storage stability test	No observations of corrosion or packaging degradation during 1-year storage stability study.
830.7000	pH	Not applicable, the TGAI/MUP is a powder	Not applicable, EP is a powder
830.7100	Viscosity	Not applicable, per 40 CFR § 158.2120(d)(4) - TGAI/MUP is not a liquid	Not applicable, per 40 CFR § 158.2120(d)(4)
830.7300	Density/Relative Density/Bulk Density	0.280 g/cm^3	0.286 g/cm^3

TABLE 3. Tier I Toxicology Data Requirements for *Beauveria bassiana* strain ANT-03 TGAI and Bioceres EP (40 CFR § 158.2140)			
Harmonized Guideline Number	**Data Requirement**	**Results Summary and Classification**	**MRID No.**
885.3050	Acute Oral Toxicity/ Pathogenicity - Rat	Not toxic or pathogenic orally at 1.3 x 10^9 CFU/animal	48974203
885.3150	Acute Pulmonary Toxicity/ Pathogenicity - Rat	Not toxic, infective or pathogenic when inhaled at 1.1 x 10^9 CFU/animal	48974204
885.3200	Acute Intraperitoneal Injection Toxicity/ Pathogenicity - Rat	Not toxic, infective or pathogenic intraperitoneally at 1.4 x 10^9 CFU/animal	48974205
885.3400	Hypersensitivity Incidents	The EPA regulations require that hypersensitivity incidents, including immediate-type and delayed-type reactions of humans and domestic animals, that occur during the testing or production of the technical grade of the active ingredient, or manufacturing-use product, or end-use product, or are otherwise known to the applicant, be reported if they occur (refer to test note #3 of 40 CFR § 158.2140(d)).	
885.3500	Cell Culture	Not required because *Beauveria bassiana* strain ANT-03 is not a virus (refer to test note #4 of 40 CFR § 158.2140(d)).	n/a
870.1100	Acute Oral Toxicity	Waived TOXICITY CATEGORY III	48974213 (TGAI) 48974302 (EP)
870.1200	Acute Dermal Toxicity	Dermal NOAEL combined > 5050 mg/Kg bw TOXICITY CATEGORY IV	48974213 (TGAI) 48974206 (TGAI and EP) 48974302 (EP)
870.1300	Acute Inhalation Toxicity	Waived TOXICITY CATEGORY III	48974213 (TGAI) 48974302 (EP)
870.2400	Primary Eye Irritation	Waived TOXICITY CATEGORY III	48974213 (TGAI) 48974302 (EP)
870.2500	Primary Dermal Irritation	Waived TOXICITY CATEGORY III	48974213 (TGAI) 48974302 (EP)

TABLE 4. Non-Target Organism Toxicology Data Requirements for *Beauveria bassiana* strain ANT-03 TGAI and Bioceres EP (40 CFR 158.2150)			
Data Requirement	**Harmonized Guideline Number.**	**Results Summary and Classification**	**MRID No.**
Avian oral toxicity/pathogenicity	885.4050	An avian oral test indicated that *B. bassiana* strain ANT-03 is not toxic or pathogenic to Northern bobwhite (*Colinus virginianus*) dosed at a 5X field application rate (2.4 x 10^8 CFU/mL), but the birds were not tested at a maximum hazard dose. **Classification: Supplemental**	48974207
Avian Inhalation Test	885.4100	Avian inhalation test was not conducted, but a waiver was submitted. The rationale provided is not sufficient, but other information indicates that there is not likely hazard that will result for the proposed uses. **Classification: Supplemental**	48974213
Wild mammals	885.4150	Wild mammal testing was not conducted, but a data waiver was requested. Studies conducted with *B. bassiana* strain ANT-03 on laboratory animals are appropriate for use in risk assessment. Studies with laboratory rats indicated no concerns for toxicity/pathogenicity in mammals. **Classification: Acceptable**	48974213
Freshwater fish toxicity/pathogenicity	885.4200	A freshwater fish test with *B. bassiana* strain ANT-03 showed no toxic or pathogenic effect on Rainbow trout (*Oncorhynchus mykiss*) dosed at 1 x 10^6 CFU/mL. However, mortality in one of the three test substance replicates was high and the replicate was discarded. Because of the above high mortality data, the 30 day LC_{50} could not be determined conclusively. **Classification: Supplemental**	48974208
Freshwater invertebrate toxicity/pathogenicity	885.4240	A freshwater invertebrate study with *B. bassiana* strain ANT-03 showed 20% mortality of *Daphnia magna* at exposure to 1 x 10^3 CFU/mL of test medium. 100% mortality was shown when tested at 1 x 10^6 CFU/mL. An EC_{50} could not be determined, but is estimated to be between 10^3 and 10^6 CFU/mL. **Classification: Supplemental**	48974209

TABLE 4. Non-Target Organism Toxicology Data Requirements for *Beauveria bassiana* strain ANT-03 TGAI and Bioceres EP (40 CFR 158.2150)

Data Requirement	Harmonized Guideline Number.	Results Summary and Classification	MRID No.
Non-target insect testing	885.4340	A laboratory bioassay with adult ladybird beetles (*Hippodamia convergens*) at 10X the field application rate showed no mortality of *B. bassiana* strain ANT-03 to the test organism (*Helicoverpa zea*) within 8 days of testing. However, reduced food consumption was observed with the test substance group during Days 7-11 when compared to the untreated control group. **Classification: Supplemental** A laboratory bioassay with green lacewing larvae (*Chrysoperla rufilabris*) at 10X the field application rate showed no adverse effects of *B. bassiana* strain ANT-03 to the test organism (*Helicoverpa zea*) within 25 days of testing. **Classification: Acceptable** A waiver from the requirement of Non-target Insect Testing for a third species was requested, but scientific rationale provided for a waiver was not sufficient. **Classification: Supplemental**	48974210 48974211 48974213
Honey bee testing	885.4380	Because *B. bassiana* is a known entomopathogenic fungus with a broad host range, scientific rationale provided was not sufficient to conclude that no adverse effects of *B. bassiana* strain ANT-03 on honey bees are likely to occur. **Classification: Supplemental**	48974213
Estuarine/marine fish and invertebrate testing	885.4280	A waiver from the requirement for the Estuarine/Marine Fish and Invertebrate Testing was requested. Scientific rationale provided was insufficient to conclude that no adverse effects are expected on non-target fish and invertebrates for all applications. *B. bassiana* strain ANT-03 was shown to be toxic to the aquatic invertebrate *Daphnia magna* at 1 x 10^6 CFU/mL. Repeated applications over large areas may lead to some exposure of *B. bassiana ANT-03* in nearby aquatic environments. **Classification: Supplemental**	48974213
Non-target plant Testing	885.4300	A data waiver from Non-target Plant Testing of *B. bassiana* strain ANT-03 was requested. Scientific rationale largely based on existing *B. bassiana* strains submitted was not sufficient to support no adverse effects to all plants. **Classification: Supplemental**	48974213

APPENDIX B. PESTICIDE PRODUCTS

EPA Registration Number	Product Name	Percentage Active Ingredient	Formulation Type	Use Site(s)	Method(s) of Application	Application Rate	Target Pest(s)
89600-1	*Beauveria bassiana* strain ANT-03 Technical	100%	Manufacturing-Use Product (powder)	For manufacturing use only	N/A	N/A	N/A
89600-2	Bioceres	20.0%	End-Use Product (wettable powder or water dispersible briquettes)	Agricultural and ornamental crops, turf, residential	Ground, aerial, chemigation	Up to 6 lbs/acre	Foliar pests (whiteflies, aphids, thrips, plant bugs, certain drosophila, beetles and borers) and certain grubs

SEMI-FIELD TRIALS TO EVALUATE THE IMPACTS OF BIOCERES WP TREATMENTS ON THE HONEYBEE'S (*APIS MELLIFERA)* HEALTH, BEHAVIOUR AND AFTER WINTERING PERFORMANCE.

Madeleine Chagnon Ph.D.

UQAM-CRSAD

May 2019

SUMMARY

In summer 2018, twelve test tunnels were put up at Le Centre de Recherche en Sciences Animales de Deschambault (CRSAD), a para-governmental experimental farm in Quebec, Canada. Two groups (Control (water) and Treatment (BioCeres) of six hives were placed in the twelve tunnels covering a red and white clover surface areas of 41,8m^2 (first trial from 21 June to 1 July 2018) to 50,6 m^2 (second trail from 13 to 23 August 2018). Water feeders were placed near hives in each tunnel. To assess acute bee mortality, dead bee traps were positioned on hives and vegetation mats were placed on tunnel floors. The bioinsecticide was applied, according to the label, when honeybees were not active, after sunset. Colonies were initially exempt of clinical symptoms of disease. Daily data collected included honeybee, drone and larvae mortality and flight and drinking activity. In hive periodic data collection included number of food stock cells (honey and pollen), number of brood cells and adults bees on frames. Meteorological data was collected hourly inside and outside tunnels during the trials. Using T-test comparisons, no statistical difference was found between treatments for all recorded data. On rare occasions where dead bee counts were greater than average (although very low), specimens from the BioCeres tunnels were sent to the laboratory for incubation and biotic analysis. Results did not show the presence of the fungal infection to *B. bassiana.* Hives were thereafter wintered inside at de CRSAD and after wintering data were registered in April 2019. Again, no statistical differences between colonies exposed to BioCeres treatments au controls were found. BioCeres WP, containing the active ingredient of conidiospores of the strain *B. bassiana* ANT-03, at the highest recommended doses did not infect, cause sub lethal behavioural abnormalities, nor kill the honey bees under tested conditions. The study results indicate that BioCeres WP is safe to honeybees under field conditions and can be applied against field pest insects.

1. INTRODUCTION

This report presents the results of the Semi-Field study trials to evaluate the short and long term impacts of BioCeres WP treatments on honeybee's, *Apis Mellifera,* heath and behaviour.

This study has been requested by the PMRA for the registration of the BioCeres wettable formulations, F WP, D WP, and BioCeres granular formulation F GR and D GR against field pest insects. The study will provide information to evaluate the effect of the active ingredient of the BioCeres, the strain of the *Beauveria bassiana* ANT-03 on the health of the colonies and the risk assessment for the pollinator (CODO M9.5.1).

The experimental protocol is based on the OECD's Guidance Document on the Honey Bee (*Apis Mellifera* L.) Brood Test Under Semi-Field Conditions (Series on Testing and Assessment Number 75, ENV/JM/MONO(2007)22) , with modifications submitted to Anatis by PMRA (document untitled: PMRA Comments to the draft protocol sent on July 24, 2017, sent by Stéphanie Girard, PMRA, Nov 7, 2017).

2. EXPERIMENTAL DESIGN AND METHODOLOGY

2.1 Tunnels

Twelve test tunnels were put up at Le Centre de Recherche en Sciences Animales de Deschambault (CRSAD), a para-governmental experimental farm in Quebec, Canada. *46°, 40.27' N; 10°71.50'O*). Tunnels consisted of car shelter structures shielded with FIINTEX knitted insect exclusion netting (1,9mmx0.095mm mesh; 60gr/m^2). Each covered a crop area of 41,8m^2 (25ft X 18ft) for the first series of trials 40 m^2 and were enlarged to 50,6 m^2 (20ft X 30ft) for the second series of trials, taking into account the colony development and a greater expected number of foragers. Tunnels were 2,74m in height. They were placed in two groups or six, sited 30 meters apart to avoid interference between treatment groups. (Figure1). The first group of six tunnels was used to expose honeybee colonies to BioCeres® WP (Figure 2) while in the six others were there control colonies. These were placed away from known sources of agricultural pesticides. One hive was placed in each tunnel, along with one water feeder for the foraging bees (10cm x 20cm). Three lightweight sponges were placed on the water surface to allow bees to land (Figure 3). The water content was refilled or replaced daily. During the applications, the water feeders were removed from the tunnels. Two mats (1m x 2m) were placed on the vegetation floor of each tunnel in order to collect supplemental dead bees.

2.2 Vegetation

The tunnel surface area was covered with red and white clover (*Trifolium pratense* and *Trifolium repens*) as well as mustard *Brassica sp.* Flowers were attractive to honey bees

and flowered during all the exposition period (Figures 4 ab). The neighboring environment on the experimental farm was free from bee attractive crops. Crop protection compounds (insecticides) are not used on this farm.

2.3 Colony preparation and exposure

Initially, colonies were free of clear clinical symptoms of disease (e.g. *Varrosis, Nosemosis, Amoehiosis,* Chalkbrood, Sacbrood, American or European foulbrood) or pests *(Varroa destructor).* Sister queens were used in order to minimize genetic variability. The healthy honey bee colonies, were placed in the tunnel tents shortly before full flowering of the crop assuring an acclimation period of at least 30 hours in the tunnel prior to the application of the test bioinsecticide (day 0). All hives were equipped with a dead bee trap at the entrance to count the number of dead bees (Figure 5).

First series of exposures started on 21 June 2018 and were continued until 1 July 2018 while the second series were from 13 August to 23 August 2018. At the end of days 0, 3 and 6 of the experimental periods, the vegetation inside each of the six the BioCeres® treatment tunnels (containing one of the experimental hives) was sprayed with BioCeres® WP at the maximum label recommended application rate (6 g/L) and a volume rate of 200-400 L/ha. In the six control tunnels, the vegetation was treated with tap water at a volume rate of 200-400 L/ha. The test product (BioCeres® WP) and the control (water) were applied using a 16 l backpack sprayer with a calibrated nozzle (Figure 6). Treatments started at sunset to avoid honeybee foragers and spraying of the insect net was also avoided. The bioinsecticide was applied, according to the label, when honeybees were not active, after sunset. Both test product and water were applied during the same time period. Honeybees foraged the treated vegetation on the next morning. Following the full first honeybee exposure period in the tunnels, the hives were placed outside their respective tunnel and were free to forage in the field.

The second series of treatments started on 13 August 2018 and were continued until 23 August 2018. Experimental design was identical to that of the first series of treatments. The hive identification numbers and treatment codes are listed in Table 1. The same hives were attributed to the same tunnel/treatments for the two series.

After the final November 2018 data collection on hives from both treatments (BioCeres and Control (water), colonies were wintered in a heated and ventilated indoor wintering facility at le Centre de Recherche en Sciences Animals de Deschambault (CRSAD). In April 2019, hives were taken out of hibernation for final evaluations

2.4 Statistical analysis

Statistical analysis was performed using JMP Software (SAS). Fisher F test were used when variance was analyzed and two independent factors were considered and a Student t test was used to check difference between means when only two dependent variables and sample numbers were low. When data lines were higher a Z-test was performed.

3. DATA COLLECTED

3.1 Colony health and food stocks

On 20 June 2018, prior to the first series of trials, the colonies were assessed for health and food stocks. The same was done on 10 August prior to the second series of trials as well as on 4 September, at the end of the second series of trials. Each frame was removed and inspected one at a time, data regarding number of adult bees and surface measurements of nectar, pollen, eggs, open brood and capped brood was recorded. After the first treatment period, the strength of the colony (through estimation of comb area covered with bees), the presence of a healthy queen, and adequate comb areas with pollen and nectar were assessed. A last complete pre hibernation frame evaluation was done on 4 September 2018. Before and after wintering, two final data collections (November 2018 and April 2019) were done on colony (adults and brood) populations, colony weight, number of frames with bees, food stocks, and syrup consumption,

3.2 Behavioural data

During testing, flight activity, foraging activity and drinking activity was recorded every day or second day (occasionally). On a 1 m^2 area, at three different places in each tunnel, the number of bees that were foraging on flowering plants was counted for a short time period (under 60 seconds). At the same time, the number of open flowers in the observed $1m^2$ of vegetation were also counted. The precision of surface area was assured by placing a $1m^2$ wooden square over the vegetation. Bees flying around the crop were also counted. Those flying restlessly or fixed to the tunnel surfaces were omitted from this count, their behavior being recognized to be due to confinement conditions. Number of honeybees drinking in the water feeders was also recorded.

3.3 Mortality data (Dead bee traps and vegetation mats)

The assessments of the number of dead bees was carried out at approximately the same time as honey bee flight observations, at the end of the morning using dead bee traps (Figure 5). Collected dead bees were counted and numbers placed in one of three categories: foragers, drones and larvae.

After first testing period, on 16. 23 and 31 July (between the two series of testing), mortality of adult bees, drones and larvae were assessed in dead bee traps, once a week. The same was done after the second testing period on 30 August and 7 and 12 September 2018.

At the same occasion, numbers of dead honeybees on vegetation floor mats were registered and thereafter removed.

3.4 Meteorological data

Temperature, relative humidity and rainfall were recorded at two paralleled stations, one inside a tunnel (Figure 7) and one outside the tunnels. Average, minimum and maximum temperature and relative humidity were then calculated. Data from the two stations were

compared. Rainy periods were avoided for the performance of the test. Wind speed and cloudiness (solar radiation) were also assed but were deemed null during the test product and water spaying, after sunset, in accordance with label guidelines.

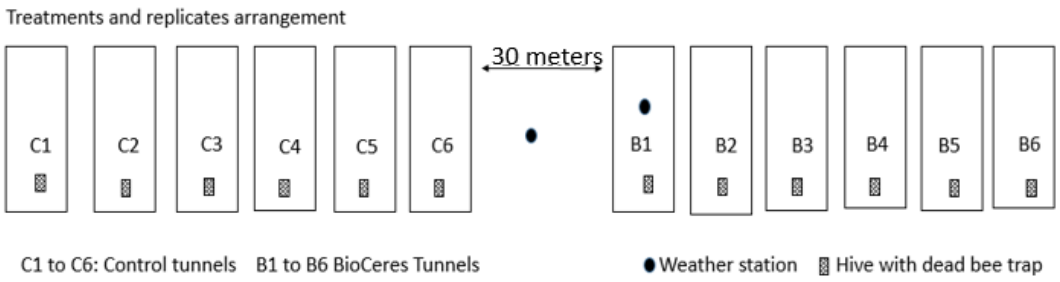

Figure1. Experimental design

Figure 2. Six control tunnels and six BioCeres® WP test tunnels separated by a 30 m distance to avoid interference. Deschambeault, Québec, Canada, June 2018.

Table 1. Treatment codes and hive identification numbers.

BioCeres		**Water (control)**	
Code	Hive number	Code	Hive number
B1	87	C1	93
B2	70	C2	52
B3	78	C3	64
B4	47	C4	63
B5	50	C5	82
B6	68	C6	80

Figure 3. Water feeder (10cmx20cm) with three light sponges floating on water were placed into each tunnel. Deschambault, June 2018.

Figure 4. Honeybee foraging on red clover (*T. pratense*) and white clover (*T. repens*).

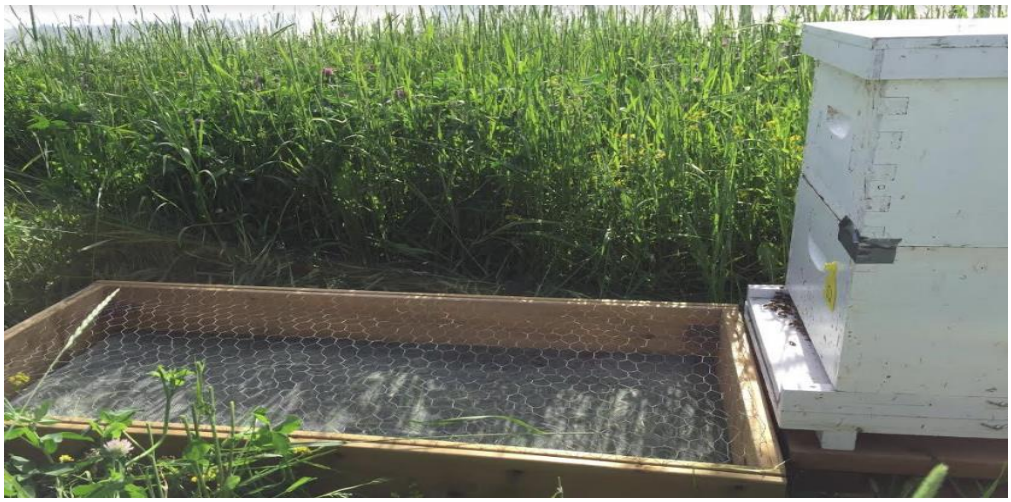

Figure 5. Dead bee traps were placed in front of each hive.

Figure 6. The test product (BioCeres® WP) and the control (water) were applied after sunset using a 16 l backpack sprayer.

Figure 7. Temperature, relative humidity and rainfall were recorded inside a tunnel and compared to the same data outside the tunnels

4. SUMMER 2018 RESULTS

4.1 Evaluation on hives

4.1.1 Colony health

For all these data, the bilateral critical value of Student t equals 2,229 at α=0, 05 and d.f. =10.

Before the first testing period (20 June) (Table 2), no statistical difference was found between the six hives in the BioCeres and the Control (water) tunnels for the number of bees on the frames (t=0,55; d.f.=10; P=0,58) and number of open brood cells (t=0,45; d.f.=12; P=0,6924). The mean number of bees on the frames being 24 300 for the BioCeres hives and 23167 for the control hives. There was a significant difference between the number of capped brood cells (t=2,8; d.f.=10; P= 0.0186). The mean number of capped brood cells in the BioCeres hive frames was 14 527 and 17 674 in the control hive. However, when total brood cells (open and capped together) were compared, difference is considered to be not statistically significant (t=1, 46, d.f. =10; P= 0, 1741).

The day after the end of the first treatment period, the strength of the colony (through visual estimation of comb area covered with bees), the presence of a healthy queen, and adequate comb areas with pollen and nectar were assessed and found customary.

Before the second testing period, on 10 August, there was no statistical difference between BioCeres and water (Control) treatments in terms of the number of bees on frames (t=1, 26; d.f.=10;P=0,233),the number of cells of open brood (t=0,4341; d.f.=10; P=0,6734) and the capped brood cells (t=0, 3171; d.f.=10; P=0,7578) (Table 3).

After the second and last testing period (Table 4), on 4 September, no statistical difference was found between number of bees on the frames (t= 1,268;d.f.=10; P=0,2333), the number of cells of open (t=0,4341; d.f.=10; P=0,6734) and capped brood (t=1,27; d.f.=10; P=0,7578) for hives place in BioCeres and Control tunnels. This was confirmed by an one way ANOVA (F=2,14; d.f.=11;P=0,17).

Table 2. Adult and Brood evaluations (number of cells) on 20 June 2018, before first series of treatments. Deschambault, Québec, Canada.

Hive number	Treatment	Adult bees	Capped brood	Open brood	Total brood
70	BioCeres	21400	15661	13179	28840
47	BioCeres	31100	11779	4026	15805
68	BioCeres	21300	17344	7341	24685
50	BioCeres	26500	17227	4193	21420
87	BioCeres	21000	11423	19812	31235
78	BioCeres	24500	13725	15355	29080
Mean		**24300**	**14527**	**10651**	**25178**
SD		**3986**	**2621**	**6465**	**5789**
63	Water	21200	17059	15861	32920
64	Water	20300	17954	12116	30070
52	Water	24400	16444	6936	23380
93	Water	23300	17837	10498	28335
82	Water	28400	18832	8363	27195
80	Water	21400	17919	16461	34380
Mean		**23167**	**17674**	**11706**	**29380**
SD		**2971**	**824**	**3884**	**3997**

Table 3. Adult and Brood evaluations (number of cells) on 10 August 2018, before second series of treatments. Deschambault, Québec, Canada.

Hive number	Treatment	Adult bees	Capped brood	Open brood	Total brood
70	BioCeres	24300	17142	10768	27910
47	BioCeres	15000	14027	7954	21980
68	BioCeres	21700	15850	16360	32210
50	BioCeres	20600	0	5280	5280
87	BioCeres	26700	21682	14893	36575
78	BioCeres	14900	15368	11647	27015
Mean		**20533**	**14011**	**11150**	**25162**
SD		**4815**	**7351**	**4153**	**10921**
63	Water	23100	17615	8105	25720
64	Water	16500	16312	12388	28700
52	Water	23100	16309	13561	29870
93	Water	21650	16194	11771	27965
82	Water	20600	6694	15526	2220
80	Water	17300	11638	13503	25140
Mean		**20375**	**14127**	**12476**	**23269**
SD		**2864**	**4181**	**2496**	**10466**

Table 4. Adult and Brood evaluations (number of cells) on 4 September, after second series of treatments.

Hive number	Treatment	Adult bees	Capped brood	Open brood	Total brood
70	BioCeres	13500	4335	9485	13820
47	BioCeres	22150	5672	11288	16960
68	BioCeres	15000	3497	11323	14820
50	BioCeres	13400	13046	12714	25760
87	BioCeres	27200	10216	17954	28170
78	BioCeres	15900	4883	13738	18620
Mean		**17858**	**6942**	**12750**	**19692**
SD		**5596**	**3808**	**2929**	**5925**
63	Water	22200	6138	11243	17380
64	Water	16700	4065	13315	17380
52	Water	27500	10747	13383	24130
93	Water	27800	5937	16174	22110
82	Water	24200	6694	15526	22220
80	Water	13700	4587	10748	15335
Mean		**22017**	**6361**	**13398**	**19759**
SD		**5758**	**2366**	**2187**	**3509**

4.1.2 Honey and pollen cells

At the beginning of our experiments, on the 20 June evaluation, the difference in number of honey cells in the BioCeres and Control (water) comb boxes was considered to be not statistically significant. (t=0, 3024; d.f. =10; P=0, 70) (Table 5). This was also true for the number pollen cells (t=1,2494; d.f.=10; P=0,24). This result was confirmed using a one way ANOVA for honey (F=0,15;d.f.=11;P=0,70) and pollen (F=1,56;d.f.=11;P=0,23) cells in comb boxes of hives from BioCeres and Control tunnels.

Table 5. Number of honey and pollen cells in hives from BioCeres and Control (water) tunnels on 20 June 2018 at the beginning of the experimental period. Deschambault, Québec, Canada.

BioCeres			**Control (water)**		
Hive number	**Honey**	**Pollen**	**Hive number**	**Honey**	**Pollen**
70	17620	3360	63	12560	2440
47	23755	1780	64	17620	2200
68	21530	3300	52	17910	4565
50	23980	8560	93	24190	2600
87	20540	2520	82	20650	1960
78	15580	3180	80	24635	540
Mean	**20501**	**3783**	**Mean**	**19594**	**2384**
SD	**3355**	**2417**	**SD**	**4558**	**1298**

After the first series of treatments and before the second series, on 10 August, the difference in number of honey cells in the BioCeres and Control (water) comb boxes was still considered to be not statistically significant (t=0,2948; d.f.=10; P=0,7742) as well as the number pollen cells (t=0,1699; d.f.=10; P=0,8685). After the second series of treatments, on 4 September, the last evaluation still showed that there was no statistical difference between honey cells and pollen cells (t=0,8832; d.f.=10; P=0,3979) (Figure 8).

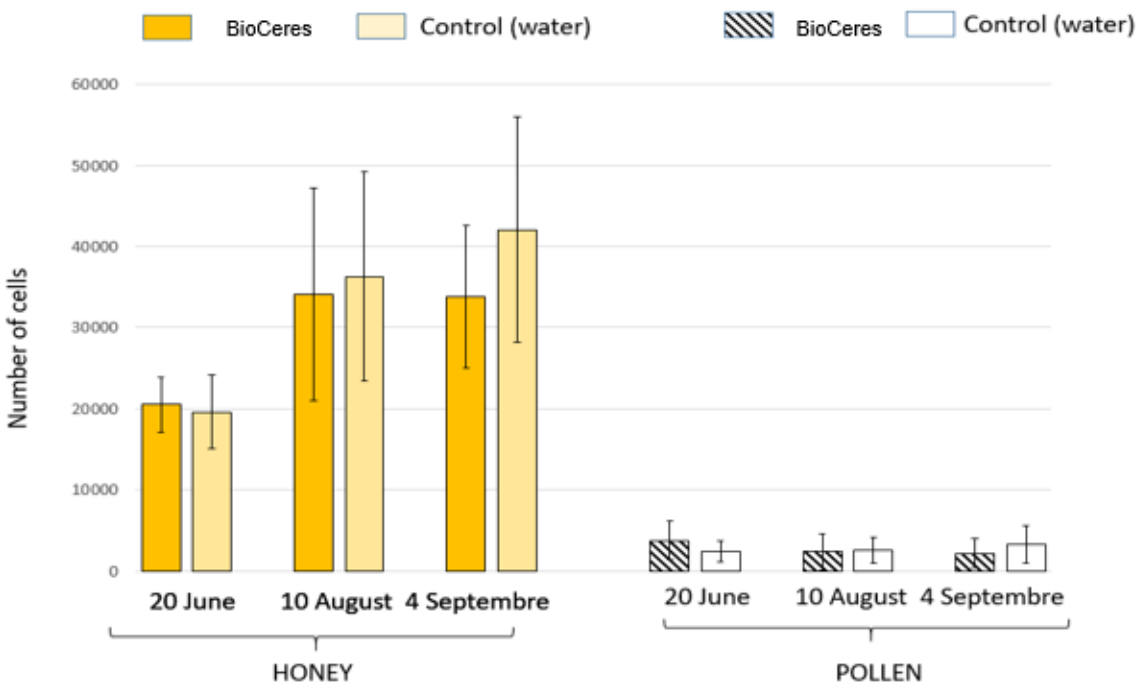

Figure 8. Mean number of honey and pollen cells in hives from BioCeres and Control (water) tunnels on 20 June, 10 august and 4 September. Deschambault, Québec, Canada.

4.2 Behavioral data

4.2.1 Foraging

First and second testing periods (20 June to 1 July 2018 and 13 to 23 August 2018).

A two way ANOVA showed a difference among treatments for number bees foraging on flowers (F=66,4; d.f.=9,; P= 7,09E-15) and also a significant difference concerning days (F=11,88; d.f.=9,;P= 1,52E-12). An interaction between these independent variables also was found. Our data on number of open flowers explains this difference. It was found that the BioCeres tunnels had, on average, less open flower per square meter than the control tunnels. (F=19,266; d.f.= 9; P=5,084E-26). The mean values were 23.95 ± 5.88 (Mean ± SD) flowers/m^2 for BioCeres tunnels and 31, 06 ±5, 78 flowers/m^2 for the control tunnels. Transforming the data to number of foraging bees per open flower gives us the same comparison picture. We now have a mean of 0,125± 0,13 foragers per flower for the BioCeres tunnels and 0,158±0,12 foragers per flower for the Control tunnels. Statistics (F-test) showed a difference between treatments (F=1,04; d.f.=170; P=0,0379) and the two-way ANOVA still show a marginal significant difference (F=11,022; d.f.=9. P=0,001). By comparing means of complete data instead of variances between hives of the same treatment, a Student t-test also confirms this statistically significant difference

(t=2, 47; d.f. = 358; P=0,0138). The critical value of t is here 1,65. In very short observation periods (a few seconds) a punctual bias can occur. Nonetheless, our data shows that honeybees in tunnels of both treatments were foraging in a similar fashion. (Figure 9). For the second testing period, only a Student t-test was performed. Here the difference between the two sets of data is considered to be not statistically significant. t=1,26; d.f. =322; P=0,2074). (Figure 10)

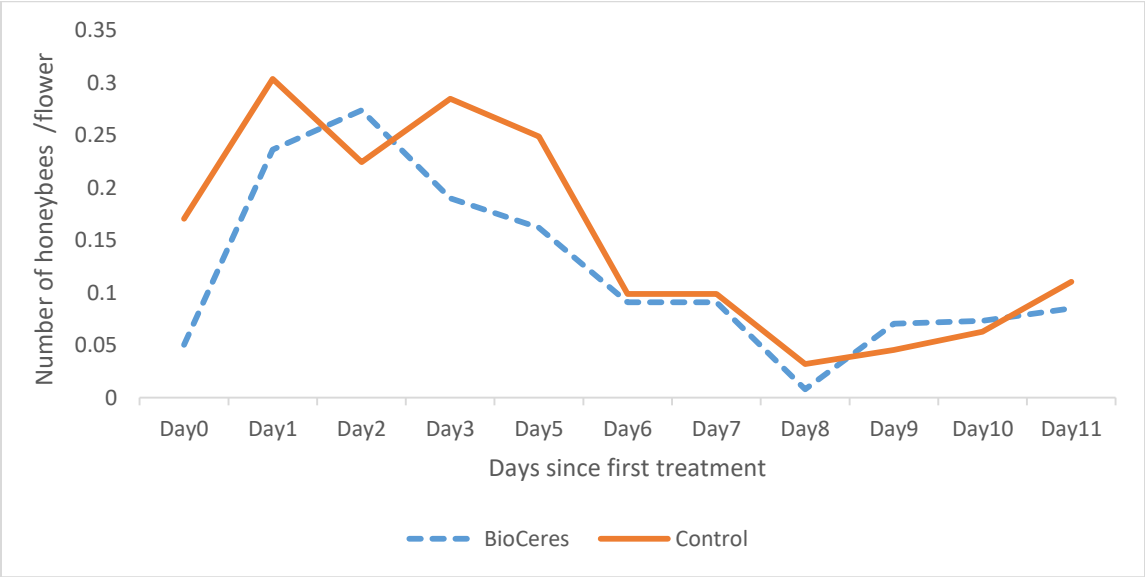

Figure 9. Mean number of honeybees per flower per m^2 at three different places in the BioCeres and Control tunnels. 21 June to 1 July 2018. Deschambault. Canada.

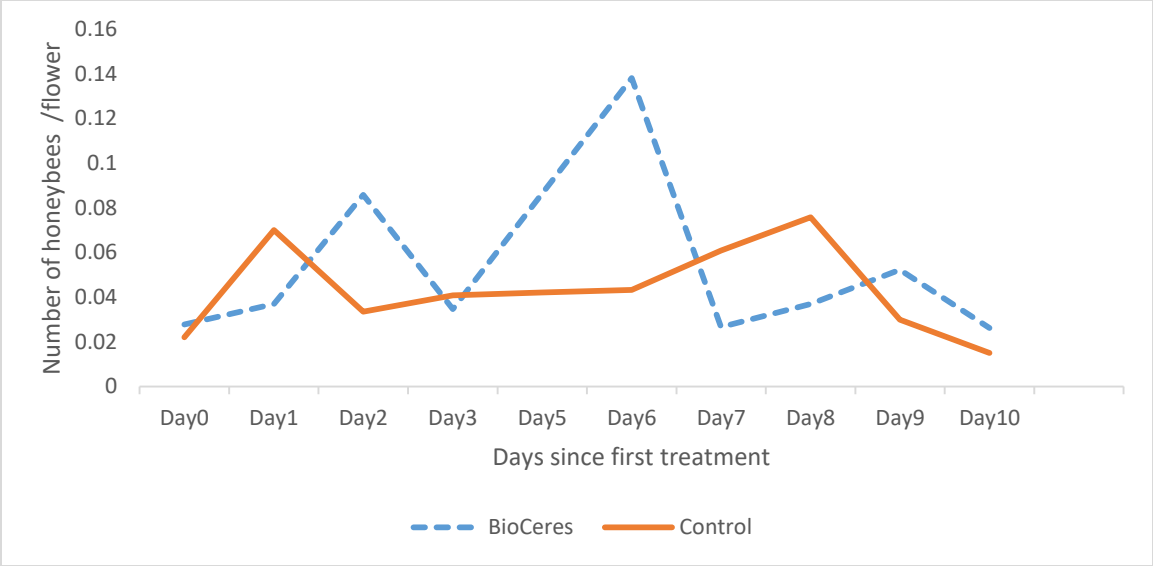

Figure 10. Mean number of honeybees per flower per m^2 at three different place in the BioCeres and Control tunnels. 13 to 23 August 2018. Deschambault. Canada.

4.2.2 Flight activity

First and second testing periods (20 June to 1 July 2018 and 13 to 23 August 2018).

No difference between number of honeybees flying between flowers was found among treatments during the first trial using a t-test (t=1,145, d.f. 106; P=0,2548) and a Fisher test (F=0,014; d.f.=9,;P= 0,9) but a significant different was found concerning days (F=11,88; d.f.=9,;P= 1,52E-12). (Figure 11). The same was found during the second trial, the difference between the two sets of data not being considered to be statistically significant (F=1,33;d.f.=106; P=0,2547) (t=1,14; d.f.=106; P=0,25) (Figure 12).

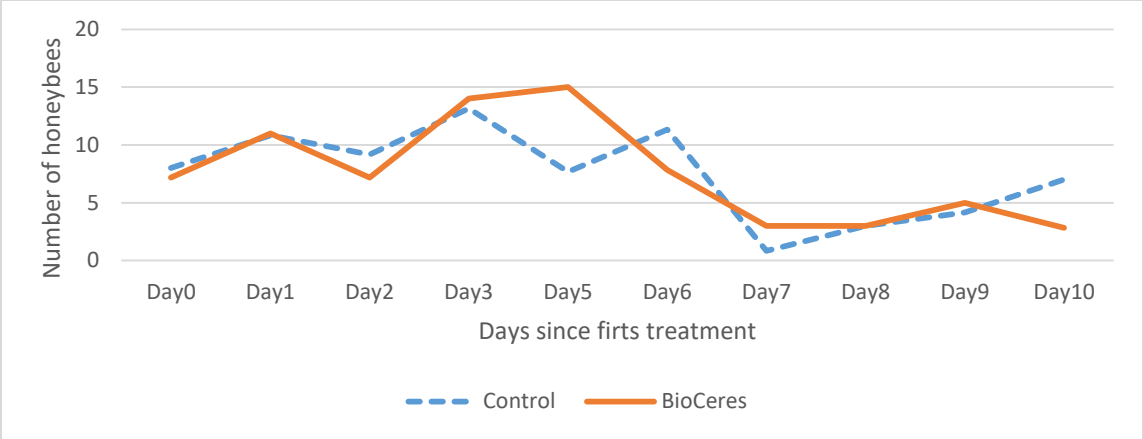

Figure 11. Mean flight activity of honeybees per m^2 (between flowers) at three different places in the BioCeres and Control tunnels from June 21 to 1 July 2018. Deschambault. Canada.

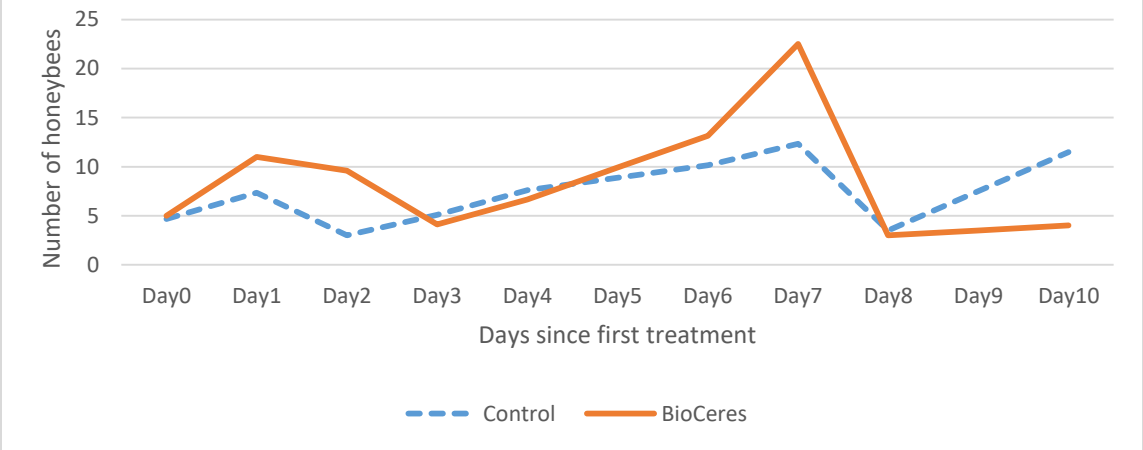

Figure 12. Mean flight activity of honeybees per m^2 (between flowers) at three different places in the BioCeres and Control tunnels from 13 to 23 August 2018. Deschambault. Canada.

4.2.3 Drinking activity

First and second testing periods (20 June to 1 July 2018 and 13 to 23 August 2018).

No difference between number of honeybees drinking in water feeders was found among treatments during the first trial using a ANOVA (F=0,54; d.f.=106; P=0,4611) and a Student t-test (t=1,145, d.f. 106; P=0,2548) (Figure 13). The same was found for the second trial period (t=0,46; d.f.=106; P=0,4611) (Figure 14).

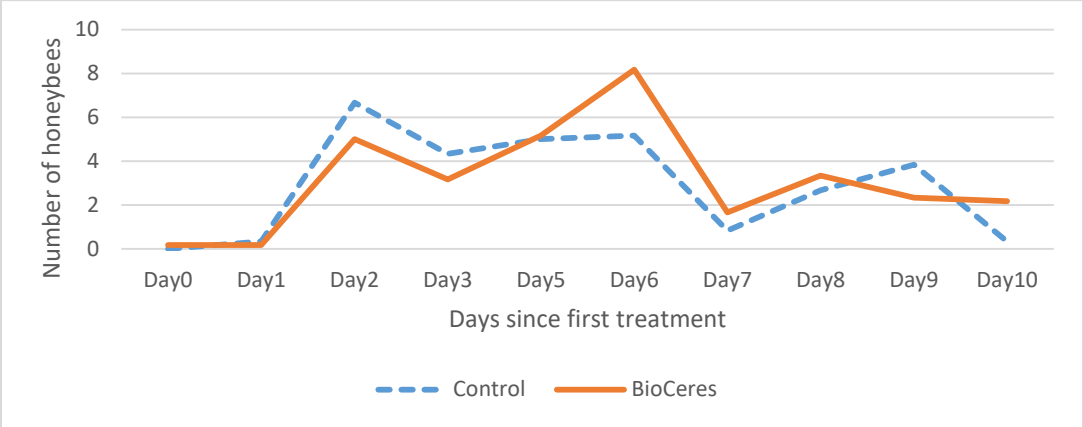

Figure 13. Mean number of honeybees drinking in water suppliers at three different places in the BioCeres and Control tunnels. June 21 to 1 July 2018. Deschambault. Canada.

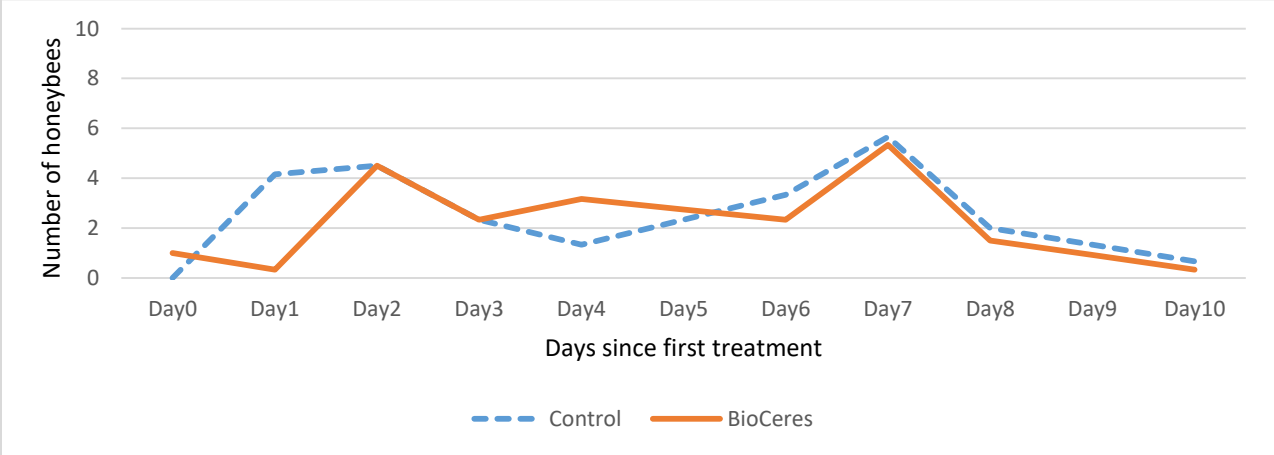

Figure 14. Mean number of honeybees drinking in water suppliers at three different places in the BioCeres and Control tunnels. 13 to 23 August 2018. Deschambault. Canada.

4.4 Mortality (dead bee traps)

Collected dead bees and larvae were counted and placed in one of three categories: adult bees, drones and larvae. Results of specimen counts for these three categories are summarized in table 6 and also illustrated in detail in figures 15 and 16.

First testing period (20 June to 1 July 2018).

A two way ANOVA was used to compare means of dead bee counts across the independent variables 'Treatment' and 'Days since the beginning of the exposure trial' (BioCeres application). Although the factorial analysis showed no interaction between independent variables, a significant difference was found between days since the beginning of the treatments for forager bees (F=3,32; d.f.119;P=0,0013), drones (F=4,45; d.f.119;P=6,4E-05) and larvae (F=7,27; d.f.119;P=4,4E-08). In fact, a much higher number of dead drones were found in dead bee traps for both treatments of the first testing period, from day 2 to day 10 (especially on days 6 to 8) (Figure 15). Dead adult bees ranged between 0 and 14 individuals. Nonetheless, on the whole, no statistical difference in number of dead specimens collected (for all three categories) was found should it be for number of adult bees (F=0,15;d.f.=119;P=0,699), drones (F=3,00;d.f.=119 ;P=0,855) or larvae (F=0,104; d.f.=9 ;P=0,746). (Table 8)

A series of Student t tests confirms these similarities. For adult bees, the difference in mortality counts between BioCeres and Control treatments is considered to be not statistically significant (t=0,39; d.f.=118; P=0,7). The same was found for drones (t=1,73; d.f.=118; P=0,08) and larvae (t=0,32; d.f.=118; P=0,3234). (Figure 15)

Table 6. Means and standard deviations of number of specimens collected daily in dead bee traps from BioCeres and Control (water) tunnels from 21 June to 1 July and from 13 to 23 August 2018. Deschambault, Quebec, Canada.

Period	Stage	Tunnels			
		BioCeres		Water (control)	
		mean	SD	mean	SD
First testing	adults	2,30	2,72	2,10	2,93
21 June to 1 July	larvae	3,45	5,73	3,82	6,66
	drones	8,08	7,93	5,78	6,54
Second testing	adults	3,11	4,82	1,78	2,22
13 to 23 August	larvae	4,63	7,25	4,26	6,18
	drones	4,20	5,35	2,44	2,21

Second testing period (13 to 23 August 2018).

Using a one way ANOVA (comparing variances), no statistical difference in number of dead specimen collected in BioCeres and Control tunnels was found for forager bees (F=3.41;d.f.=106; P=0,067) and larvae (F=0,08; d.f.=106 ; P=0,77). There was a difference between the two treatments for the number of dead drones (F=4,99; d.f.=106 ;P=0,027). As for the first testing period, there was a difference between days since the beginning of the treatment periods for drones (F=6,8; d.f.= 8; P=0,01) but also for adult bees (F=2,9;d.f.=8; P=0,006)(Figure 16). The highest number of dead adult bees

collected was on day 7 where 23 adult bees were collected. This number is considered very small, but even so, it was the highest number of adult bees collected. Therefore, the specimens were sent to the laboratory for incubation and biotic analysis. Results did not show the presence of the fungal infection by the *Beauveria bassiana*, the active ingredient of the BioCeres (Table 7).

Table 7. Mycosis development from bee's cadavers from second testing period (13 to 23 August 2018).

Hive number	Treatment	Total dead bees	Mycosis development (*B. bassiana*)*	
			number of insect with external fungal growth	growth/total number
70	BioCeres	25	0	0
78	BioCeres	17	0	0
47	BioCeres	18	0	0

*The bees were incubated at 27 C during 6 days in the moist chamber

Using a two tailed t-test to compare means, the difference between number of dead adult bees is considered to be not quite statistically significant, but still in accordance with α=0,05 (t=1,84; d.f.=106; P=0,0675; critical t: unilateral= 1,65; bilateral=1,98). For the drones, a significant difference was found (t=2, 2345; d.f.=106; P=0,0276), as in the F-test. For larvae, differences were not considered to be significantly different (t=0,2857; d.f.=106; P=0,7756).

Weekly mortality counts after testing periods

Complete accumulated weekly data from dead bee traps from hives from the Control tunnels and the BioCeres tunnels (after first and second testing periods) were not found to be statistically different for adult bees (t=0,47; d.f.=70; P=0,63), drones (t=1,87; d.f.=70; P=0,0654) and larvae (t=10,126; d.f.=70; P=0,9). (Table 8). When weekly counts were greater than average, specimens from the BioCeres tunnels were sent to the laboratory for incubation and biotic analysis. Results did not show the presence of the fungal infection to *B. bassiana* for the first (Table 9) nor the second (Table 10) testing periods.

Table 8. Means and standard deviations of specimens collected weekly in dead be traps from BioCeres and Control tunnels after first (16, 23, 31 July) and after second testing periods (20 August, 7 and 12 September. 2018. Deschambault, Quebec, Canada

Period	**Stage**	**Tunnels**			
		BioCeres		Water (control)	
		mean	SD	mean	SD
After first testing	adults	108,67	41,87	122,67	78,45
21 June to 1 July	larvae	0,44	0,78	0,5	1,65
	drones	3,83	4,64	2,33	3,76
After second testing	adults	73,22	38,33	46,44	23,71
13 to 23 August	larvae	0	0	0	0
	drones	6,89	12,35	1,94	5,15

Table 9. Mycosis development from bee's cadavers from first testing period of 21 June to 23 1 July 2018.

Hive number	Treatment	Total death bees	Mycosis development (*B. bassiana*)*	
			number of insect with external fungal growth	growth/total number
87	BioCeres	53	0	0
70	BioCeres	49	0	0
78	BioCeres	95	0	0
47	BioCeres	130	0	0
68	BioCeres	57	0	0
64	Water (Control)	42	0	0

*The bees were incubated at 27 C during 6 days in the moist chamber

Table 10. Mycosis development from bees' cadavers from weekly counts after testing second period of 13 to 23 August.

Hive number	Treatment	Total death bees	Mycosis development (*B. bassiana*)*	
			number of insect with external fungal growth	growth/total number
70	BioCeres	158	0	0
78	BioCeres	94	0	0
47	BioCeres	103	0	0
68	BioCeres	70	0	0

*The bees were incubated at 27 °C during 6 days in the moist chamber

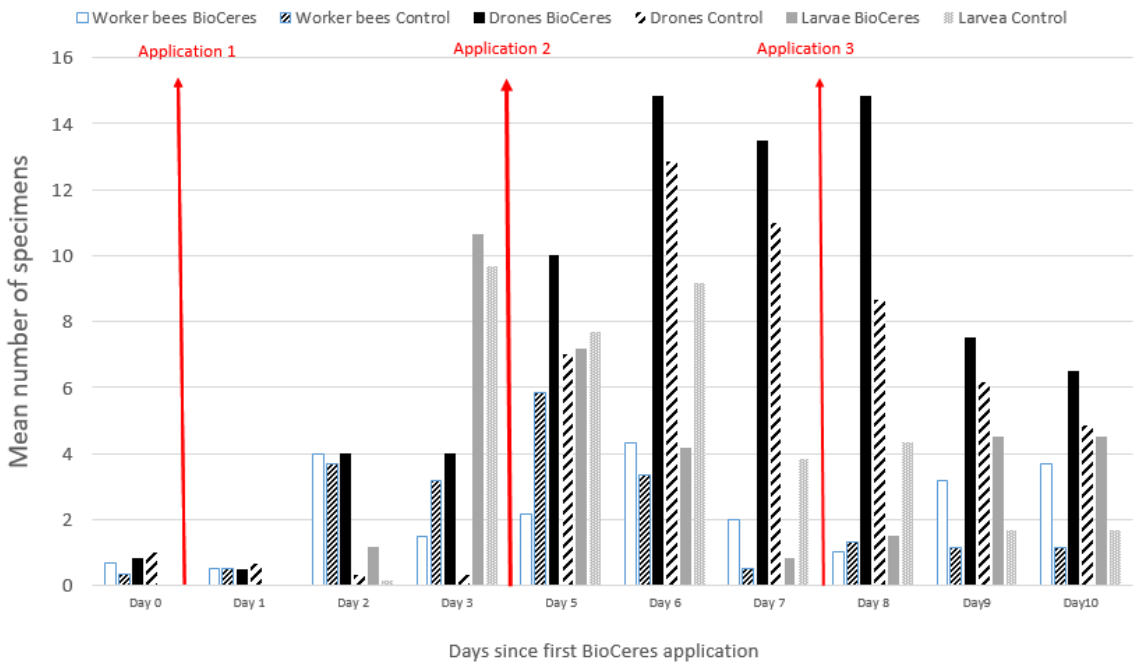

Figure 15. Daily mean number of dead specimens found in dead bee traps on hives of BioCeres and Control tunnels from 21 June to 1 July 2018. Deschambault, Canada.

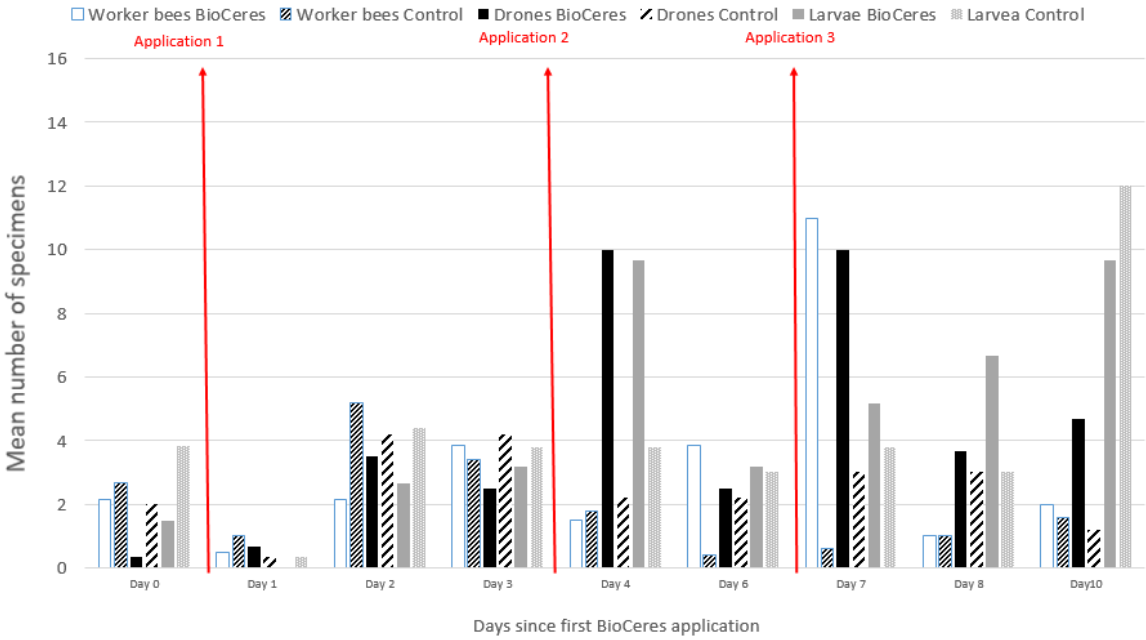

Figure 16. Daily mean number of dead specimens found in dead bee traps on hives of BioCeres and Control Tunnels from 13 to 23 August 2018. Deschambault, Canada,

4.5 Mortality (dead bees on mats)

During the first trial period the average number of dead bees found on the mats was 1,38±2,13 (mean ± SD)/day for the BioCeres tunnels and 1,18±2,11/day in the control tunnels. During the second trial period the average number of dead bees found on the mats was 1,90 ±2,70/day for the BioCeres tunnels and 1,95±2,27/day in the control tunnels.

Using a two tailed t-test to compare means, the difference between numbers of dead adult found on the two vegetation mats placed in tunnels of the BioCeres and Control treatments was not statistically significant for both the first (t=0,50, n=54. d.f.=53, P=0,6145) (and the second (t=0,038, n=54. D.f.=53, P=0,9693) trial periods.

4.6 Meteorological data

Similarities were found between data taken inside and outside the tunnels, for both testing periods (Table 11). Comparisons were not statistically different between solar radiation (Wat/m^2) (Z=13,31; n=1632; P=0) temperature (°C) (Z= 4.303 ; n= 1632; P=1,68) and relative humidity (%)(Z= 0,00032 ; n= 1632; P=1,95) for the first period and also for the second testing period: solar radiation (Wat/m^2) (Z=10.82; n=1500; P=0) temperature (°C) (Z= 0,19 ; n= 1500; P=1,95) and relative humidity (%)(Z= 0,019 ; n= 1500; P=1,96). Day by day compilation (average, minimum and maximum) of temperature and relative humidity data can be found in tables 12 and 13.

Table 11. Comparison of data between first and second testing periods, inside and outside the tunnels, from June 21 to 1 July and 12 to 23 August 2018. Deschambault, Quebec, Canada.

Site	Test period	Temperature (°C)			Relative humidity (%)		
		average	min	max	average	min	max
inside tunnel	first	18.17	4.3	33.3	74.04	19	100
	second	19.87	13.40	27.42	76.25	45.46	97.00
	difference	1.70	9.10	-5.88	2.21	26.46	-3.00
outside tunnel	first	17.77	4	31.6	74.92	9.5	100
	second	19.36	13.39	26.16	77.84	48.88	96.90
	difference	1.59	9.39	-5.44	2.92	39.38	-3.10

Table 12. Daily temperature and relative humidity at two paralleled stations, one inside a tunnel and one outside the tunnel during the first treatment period from 20 June to 1 July. Deschambault, Québec, Canada.

Day	Date	Tunnel	Temperature (°C)			Relative humidity (%)		
			average	min	max	average	min	max
0	2018-06-20	inside	18.85	19.90	23.10	75.34	55.40	90.20
		outside	19.10	12.10	28.70	73.93	37.60	91.50
1	2018-06-21	inside	16.12	5.80	33.30	52.30	19.00	89.00
		outside	15.42	5.30	31.60	53.27	9.50	91.50
2	2018-06-22	inside	15.41	4.30	24.60	66.50	30.40	100.00
		outside	15.38	4.30	24.00	66.91	30.40	100.00
3	2018-06-23	inside	17.93	9.30	25.60	73.75	45.80	97.30
		outside	16.69	9.40	24.90	75.97	51.09	97.10
4	2018-06-24	inside	16.75	12.30	22.90	82.28	59.20	97.90
		outside	16.25	12.50	21.30	83.91	62.10	97.90
5	2018-06-25	inside	15.90	8.90	24.70	66.71	27.60	99.50
		outside	15.40	8.60	23.30	66.34	23.50	98.70
6	2018-06-26	inside	15.94	5.10	24.20	64.00	29.40	100.00
		outside	15.81	4.90	23.70	63.35	28.30	100.00
7	2018-06-27	inside	19.47	8.10	28.20	64.36	33.40	97.80
		outside	19.22	8.20	27.60	65.17	32.50	97.10
8	2018-06-28	inside	19.22	14.50	23.70	88.19	68.00	99.40
		outside	18.86	14.40	22.90	88.26	70.00	99.50
9	2018-06-29	inside	20.59	14.40	28.50	86.00	55.50	100.00
		outside	20.14	14.10	27.60	87.53	59.00	97.90
10	2018-06-30	inside	20.35	15.40	27.60	87.52	66.10	100.00
		outside	20.91	15.20	26.80	89.26	69.00	100.00
11	2018-07-01	inside	20.97	16.00	27.10	82.51	64.10	98.70
		outside	21.00	15.60	26.00	85.67	70.90	98.70
Complete data		inside	18.17	4.30	33.30	74.04	19.00	100.00
		outside	17.77	4.00	31.60	74.92	9.50	100.00

Table 13. Daily temperature and relative humidity at two paralleled stations, one inside a tunnel and one outside the tunnel during the second treatment period from 13 – 23 August2018. Deschambault, Québec, Canada.

Day	Date	Tunnel	Temperature (°C)			Relative humidity (%)		
			average	min	max	average	min	max
0	2018-08-13	inside	21.93	13.70	32.10	75.31	36.00	99.50
		outside	21.47	13.30	30.80	77.10	39.10	100.00
1	2018-08-14	inside	23.80	16.80	32.80	77.48	46.00	97.20
		outside	23.27	16.80	30.80	79.35	49.40	97.10
2	2018-08-15	inside	21.47	13.90	27.30	83.90	61.10	99.50
		outside	21.08	13.70	26.00	85.22	65.00	99.50
3	2018-08-16	inside	17.25	11.20	25.40	71.66	38.20	97.90
		outside	16.67	11.30	22.90	72.35	42.00	97.10
4	2018-08-17	inside	16.63	9.40	24.90	86.50	49.10	99.70
		outside	16.26	9.80	24.00	87.61	56.50	99.50
5	2018-08-18	inside	19.82	14.70	27.20	77.24	49.60	97.50
		outside	19.30	14.80	26.00	79.10	51.90	97.90
6	2018-08-19	inside	19.92	13.80	28.20	70.94	34.00	98.80
		outside	19.18	14.10	26.40	72.66	38.20	98.70
7	2018-08-20	inside	19.90	12.10	28.70	70.68	29.10	96.90
		outside	19.42	12.50	27.60	72.15	27.30	96.20
8	2018-08-21	inside	20.22	13.00	28.30	76.78	51.70	97.70
		outside	19.63	12.90	26.40	78.96	58.00	97.10
9	2018-08-22	inside	19.30	15.90	23.10	84.25	68.30	97.10
		outside	18.95	15.60	22.90	85.67	70.90	96.20
10	2018-08-23	inside	18.37	12.90	24.70	64.04	37.00	85.20
		outside	17.83	12.50	24.00	66.11	39.40	86.60
Complete data		inside	19.87	13.40	27.42	76.25	45.46	97.00
		outside	19.36	13.39	26.16	77.84	48.88	96.90

5. AFTER WINTERING (2019) RESULTS

5.1 Adult bees and brood cell numbers

Although a normal winter loss of 14% is foreseeable at the CRSAD (in Québec, Canada), all of the colonies of both treatments (12) survived. Statistical analysis using a Student T-test were performed on data collected from hives of both BioCeres and water treatments.

In April (after wintering), mean numbers of adult honeybee were not statistically different (t=0,732, N=12, d.f.=11, P=0,24) in hives from the BioCeres (10450) and the Control (water) (11225) treatments (Table 14). There was no significant difference (t=3397, N=12, d.f.=11, P=0,3690) in capped brood cell numbers in hives from the BioCeres (6608) and the Control (water) treatments (3769). Likewise, there was no significant difference (t=9138, N=12, d.f.=11, P=0,1858) in open brood cell numbers in hives from the BioCeres (4639) and the Control treatments (5798).

Number of frames with bees from the BioCeres (6,96) and water (7,29) treatments were also not significant (t=0,189, N=12, d,f, =11, P=42), neither was the winter loss compared to November 2018 data (t=0,8216, N=12, d.f.=11, P=0,42) (Table 15).

Table 14. Adult and Brood evaluations (number of cells) after wintering. Deschambault, Québec, Canada.

Hive number	Treatment	Adult bees	Capped brood	Open brood	Total brood
70	BioCeres	8600	15448	6603	8150
47	BioCeres	9800	3674	5326	9000
68	BioCeres	11850	n.a	897	n.a
50	BioCeres	9000	6131	3699	9830
87	BioCeres	11400	2552	6378	8930
78	BioCeres	12050	5236	4934	10170
Mean		**10450**	**6608**	**4639**	**9156**
SD		**1508**	**5131**	**2113**	**751**
63	Water	12600	3728	7023	10750
64	Water	8400	2580	5520	8100
52	Water	13800	5051	7179	12230
93	Water	12500	4774	3526	8300
82	Water	10800	4154	6146	10300
80	Water	9250	2329	5401	7740
Mean		**11225**	**3769**	**5798**	**9570**
SD		**2108**	**1122**	**1336**	**1796**

Table 15. Number of frames with bees before (November 2018) and after (April 2019) wintering. Deschambault, Québec, Canada.

Hive number	Treatment	November 2018	April 2018
70	BioCeres	6.00	5.25
47	BioCeres	6.25	6.50
68	BioCeres	6.25	8.50
50	BioCeres	8.00	6.25
87	BioCeres	7.00	8.25
78	BioCeres	5.75	7.00
Mean		**6.54**	**6.96**
SD		**0.83**	**1.24**
63	Water	7.25	8.25
64	Water	5.75	6.50
52	Water	6.75	8.00
93	Water	7.75	8.00
82	Water	6.50	7.25
80	Water	3.50	5.75
Mean		**6.25**	**7.29**
SD		**1.51**	**0.99**

5.2 Winter weight loss

After wintering, in April 2019, data were collected on colony weight loss. The colonies treated with BioCeres weighted on average 32,6 Kg and the colonies treated with water weighted 31,9 Kg (Table 16). There was no significant effect of treatments on loss of colony weight during winter ($t=0.25$, $N=12$, $d.f.=11$; $P= 0.40$).

Table 16. Weight (Kg) weight loss of colonies before (November 2018) and after (April 2019) wintering. Deschambault, Québec, Canada.

Hive number	Treatment	November 2018	April 2018	Lost
70	BioCeres	39.8	31.2	8.6
47	BioCeres	42.4	33.6	8.8
68	BioCeres	44.0	34.0	10.0
50	BioCeres	41.6	30.4	11.2
87	BioCeres	42.6	33.0	9.6
78	BioCeres	41.2	33.6	7.6
Mean		**41.9**	**32.6**	**9.3**
SD		**1.4**	**1.5**	**1.3**
63	Water	41.2	28.0	13.2
64	Water	42.6	35.4	7.2
52	Water	39.4	30.0	9.4
93	Water	38.6	29.0	9.6
82	Water	42.4	33.2	9.2
80	Water	42.4	35.6	6.8
Mean		**41.1**	**31.9**	**9.2**
SD		**1.7**	**3.3**	**2.3**

5.3 Food supply and syrup consumption

Number of cells with remaining food supplies (Syrup and pollen) were also statistically similar in hives from both treatments for syrup (t=0, 28, N=12, d.f.=11, P=0, 39) and for pollen (t=0,80, N=12, d.f.=11, P=0,22) (Table 17). Winter consumption of syrup was also statistically similar (t= 0,24, N=12, d.f.=11, P=0,40) with a mean of 9,3 Kg for the honeybees from the BioCeres hives and 9,2 for those from the control hives.

Table 17. Number of the cells remaining food supply (Syrup and pollen) and winter syrup consumption from BioCeres and Control hives.

Hive number	Treatment	Pollen	Syrup	Syrup consumtion
70	BioCeres	640	14400	8.6
47	BioCeres	2180	16060	8.8
68	BioCeres	2880	14480	10.0
50	BioCeres	2060	10100	11.2
87	BioCeres	1460	11420	9.6
78	BioCeres	1860	15720	7.6
Mean		**1847**	**13697**	**9.3**
SD		**752**	**2404**	**1.3**
63	Water	1620	1980	13.2
64	Water	620	21420	7.2
52	Water	1280	9620	9.4
93	Water	2000	9820	9.6
82	Water	2540	13630	9.2
80	Water	1000	20400	6.8
Mean		**1510**	**12812**	**9.2**
SD		**695**	**7332**	**2.3**

6. DISCUSSION

6.1 Colony health and food stocks

Initial colony strength comparisons (number of bees on frames, open and closed brood) in hives placed in the BioCeres and Control tunnels were shown to be similar although capped brood cells were statistically lower in hives placed in the BioCeres tunnels. This was not so after the first testing period, at the beginning of July and after the second period in September. Open and capped brood were not statistically different, showing that the BioCeres treatments did not affect the queen's egg laying behaviour. Overall, honeybee colonies in hives from the BioCeres tunnels were healthy and the queens were laying well. Only the number of bees on the frames of hives placed in the control (water) tunnels seemed higher at the end of the experiment in early September. This kind of data can be influenced by many factors influencing the number of foragers in the hive, including time of the day and weather conditions. We must keep in mind that by this type of visual evaluation, it is impossible to tell between forager bees and worker bees on the frames. Another factor influencing these results is the fact that there was swarming, probably during July, in hive number 50, placed in one of the BioCeres tunnels.

Congestion (overpopulation), heat and queen age are the main factors causing swarming. This hive had the highest number of bees on frames at the beginning of the experiment (26500) and abundant and healthy brood. The season was very warm (see meteorological data) and brood cells were high. After swarming, it is normal that the bee population drops. Data from this hive explains the lower mean population accounted for in the last hive evaluation on 4 September. Nonetheless, all hives were healthy and highly occupied.

Food stock (honey and pollen) in the comb boxes were not found to be statistically different throughout the testing periods. Honey reserves were slightly lower in the BioCeres tunnel, although not statistically different. The lower number of flowers in the BioCeres tunnels could explain this minor difference on honey resource accumulation. Honeybees were secluded in these conditions (tunnels) during 18 days of the summer resource (honey and pollen) harvest. Moreover, honey boxes were taken off of the hives during the honey extraction period, causing the honeybees to feed on the comb box resources when needed.

Alter wintering, there was no significant statistical difference in all the hive performance data (number of adult bees, colony weight, brood cells, food supplies, and syrup consumption) collected from hives of the two treatments performed.

6.2 Behaviour

Our data shows that honeybees in tunnels of both treatments were foraging in a similar fashion, although less honeybees foraging on flowers were counted in the BioCeres tunnels. It was shown that density of open flowers was lower in the BioCeres tunnels, and this could explain this difference, in spite of the fact that honeybees per flower was computed and compared for this analysis. As mentioned, in a very short observation period (a few seconds in this case) a punctual bias can occur.

The flight activity was low in both series of tunnels. Data were collected at the latest between 12h and 13h. Observations later in the day might have given higher numbers. We must nonetheless retaliate on the fact that only honeybees flying between flowers were counted and that bees flying restlessly or fixed to the tunnel surfaces were omitted from this count. Nevertheless, differences in numbers for this observation were not statistically significant and show that honeybees in the BioCeres tunnels were performing in a similar manner as those in the Control tunnels.

The number of honeybees drinking in the water feeders was also similar, showing that individual exposed to the BioCeres treatments did not have a different thirst response, which could have been triggered by an uncontrolled physiological reaction due to exposure to the treatment constituents.

The overall behaviour patterns (foraging, flight and drinking) of honeybees placed in the BioCeres and Control tunnels were not showed to be significantly different, in spite of

the fact that these observations were all punctual and could have changed from second to second.

6.3 Mortality in dead bee traps and on mats

Number of specimens collected in the dead bee traps were not statically different for adult bees. However, occasionally, more drones and larvae were collected in dead bee traps of the BioCeres tunnels during hive seclusion. This was more notable in two of the hives placed in the BioCeres treated tunnels. It was noted that these two tunnels were those with the less open flowers during both testing periods. The lack of resources could thus explain this result and lead to interpret these findings as drone eviction. During the two testing and observation periods, workers were observed evicting drones from their colony, both in BioCeres and Control tunnels. In temperate climate this usually happens in autumn. Workers stop feeding drones, deny them access to honey stores and drag them from the nest (Morse et al. 1967). The reason for this is that under starvation conditions, the cost of maintaining drones outweighs the benefits. Although this usually happens at a certain point later in the foraging season, it is known that under starvation conditions drones are removed from colony faster (Cicciarelli, 2013). We also observed a large number of drone larvae in the dead bee traps. This is also a known phenomenon as described in Langstroth (1853, p. 52): ¨The hatred of the bees extends even to the unhatched young, which are mercilessly pulled from cells and destroyed with the rest¨.

We conclude that the strength of the colonies and their seclusion in experimental tunnels must have caused this phenomena during the testing periods. Since this was observed both in BioCeres and Control tunnels, no conclusion as to the impact of the BioCeres treatment on colonies was established.

When hives were outside the tunnels, after both the testing periods, drone and larvae mortality were very low and rarely observed. Weekly mortality of adult bees, drones and larvae from BioCeres and Control treatment tunnels were not shown to be statistically different. No lagging impact of the BioCeres treatment on the exposed honeybees could thus be established.

The highest adult bee mortality (thus foragers) from the BioCeres tunnels was 27 individual. This number is to be considered insignificant in everyday apicultural practice. Nonetheless, bees were sent for further examination and laboratory analyses confirm that BioCeres spores were not present on or in these dead bees.

Number of dead bees found on the vegetation mat of both treatments were very low (usually 0) and differences between treatments were not significant for both trial periods.

6.4 Meteorological data

One of the purposes of the meteorological data collection was to verify similarities between data taken inside and outside the tunnels, for both testing periods. This was demonstrated by comparing data of two stations, one outside and one inside a BioCeres

experimental tunnel. The data were shown to not be statistically different. On day 2 of the first testing period, the temperature reached 33,3°C inside the tunnels and the relative humidity level was very low 9,5%. These relatively extreme conditions did not affect the honeybees from BioCeres, nor the Control (water) tunnels. Experimental conditions were thus suitable.

7. CONCLUSION

During the two recommended series of spring and late summer field trials, mortality of honeybees (including drones and larvae) in the BioCeres tunnels was very low and did not differ from that of the control tunnels. The honeybees' behaviour, including foraging, flight, and drinking, winter food consumption as well as the queens' egg laying performance were not affected by their exposure to the summer BioCeres treatments.

After wintering data analysis shows that colonies treated with BioCeres were similar to those treated with water as to surviving adult bees, brood cell numbers, food supplies and syrup consumption. Honeybee colonies exposed to the two series of BioCeres treatments were therefore not affected, on the short nor, the long term, by treatments.

In the light of this study, the BioCeres, containing the active ingredient of conidiospores of the strain *B. bassiana* ANT-03, at the highest recommended doses did not infect, cause sub lethal behavioural abnormalities, nor kill the honey bees under field conditions.

The current study results indicate that *B. bassiana* strain ANT-03 is safe to honeybees under field conditions and can be applied against field pest insects.

8. REFERENCES CITED

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