

***Beauveria bassiana*
strain PPRI 5339**

DOCUMENT M-MA, Section 1

IDENTITY OF THE ACTIVE SUBSTANCE

Version history¹

Date	Data points containing amendments or additions and brief description	Document identifier and version number

¹ It is suggested that applicants adopt a similar approach to showing revisions and version history as outlined in SANCO/10180/2013 Chapter 4 How to revise an Assessment Report

Table of Contents

MA 1	IDENTITY OF THE ACTIVE SUBSTANCE	4
MA 1.1	Applicant	4
MA 1.2	Producer	4
MA 1.3	Name and Species Description, Strain Characterisation	4
MA 1.3.1	Taxonomic name and strain	4
MA 1.3.2	Accession number of sample in a recognised culture collection	5
MA 1.3.3	Molecular identify of the micro-organism	6
MA 1.3.4	Indigenous or non-indigenous at the species level to the intended area of application	6
MA 1.3.5	For mutant or genetically-modified strains, indicate all known differences between the modified micro-organism and the parent wild strain(s)	6
MA 1.3.6	Include any trade names, common names, developmental code names	7
MA 1.3.7	Relationship to known pathogens	7
MA 1.4	Specification of the Material Used for Manufacturing of Formulated Products	7
MA 1.4.1	Content of the micro-organism	7
MA 1.4.2	Identity and content of impurities, additives, contaminating micro-organisms	7
MA 1.4.3	Analytical profile of batches	11

MA 1 IDENTITY OF THE ACTIVE SUBSTANCE

MA 1.1 Applicant

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MA 1.2 Producer

Please refer to confidential information submitted in Document J.

MA 1.3 Name and Species Description, Strain Characterisation

MA 1.3.1 Taxonomic name and strain

Species: *Beauveria bassiana* (Balsamo) Vuillemin

Strain: PPRI 5339

First description: Strain PPRI 5339 was originally isolated by Dr Schalk Schoeman

from the larva of a tortoise beetle, *Conchyloctenia punctate* (Coleoptera: Cassidinae) collected in Escombe, Queensburgh, KwaZulu Natal, South Africa.

Phylum: Deuteromycota
Class: *Hyphomycetes*
Order: Moniliales
Family: *Moniliaceae*
Genus: *Beauveria*

Beauveria bassiana Vuill. is a cosmopolitan, anamorph species of haploid, soil-borne Hyphomycetes. For several *B. bassiana* strains isolated in Korea and China also *Cordyceps* teleomorphs are described. *Cordyceps* is a genus of the single family Clavicipitaceae of the order Clavicipitales and it is in discussion to unify the orders Hypocreales and Clavicipitales. Therefore, several authors describe the teleomorph as a Hypocreales. Nevertheless, for none of the strains isolated in Europe or USA teleomorphs have been identified and only the asexually reproducing forms seems to exist (Rehner and Buckley, 2005).

Morphological criteria for characterisation of the species *B. bassiana* are described by de Hoog (1972). *Beauveria bassiana* is characterised by white, later yellowish or occasionally redish colonies and morphologically by its sympodial to whorled clusters of short-globose to flask shaped conidiogenous cells, which give rise to a succession of one-celled, hyaline, rarely yellowish, holoblastic conidia that are borne on a progressively elongating sympodial up to 20 µm long rachis. Conidia size range between (1.5 -) 2 - 3 (- 4) x (1.5 -) 2 - 2.5 (-3) µm.

Ongoing difficulties in applying morphologically approaches to species recognition in *Beauveria* have spurred the search for additional sources of taxonomic characters and were summarised by Rehner and Buckley (2005) (Doc M-MA 1.3.1/01). Alternative character systems to detect genetic variation within *Beauveria* include isoenzymes, chemo taxonomic characters, mitochondrial RFLP, immunological approaches, rRNA sequencing, RFLP, introns in the large subunit rDNA, RFLP and nucleotide sequences of ITS, SSCP analysis of taxon specific markers, RAPD markers and the combined use of morphology and RAPD markers.

Rehner and Buckley (2005) (Doc M-MA 1.3.1/01) used a gene-genealogical approach to investigate molecular phylogenetic diversity of *Beauveria* and presumptively related *Cordyceps* species with internal transcribed spacer (ITS) and elongation factor 1-alpha (EF1- α) sequences. Based on this approach the authors created six well-supported clades. The findings of Rehner and Buckley (2005) (Doc M-MA 1.3.1/01) supported past assumptions that *B. bassiana* is not host specific but an opportunistic entomopathogen, capable of attacking insects of a wide range of taxa.

MA 1.3.2 Accession number of sample in a recognised culture collection

The strain is deposited in the Agricultural Research Culture Collection (NRRL) International Depository Authority, 1815 N. University Street, Peoria, Illinois 61604, USA.

Accession number: NRRL 50757

MA 1.3.3 Molecular identify of the micro-organism

MA 1.3.3/01

Report: Berry, S (2012), Molecular identity of the South African *Beauveria bassiana* isolate, strain PPRI 5339. Becker Underwood, Mahogany Ridge, KZN, South Africa and Becker Underwood, NSW 2250, Australia. Report Date: 28.11.12

Guidelines: None

GLP: No

Summary

Identification of *Beauveria bassiana* strain PPRI 5339 was confirmed by sequencing of the ITS ribosomal region including the 3' end of the 18S region, the ITS1 region, the 5.8S region and the ITS2 region.

The resulting sequence was BLASTED against sequences deposited in GenBank (<http://www.ncbi.nlm.nih.gov/Blast>). Results showed that the isolate PPRI 5339 was most related (99-100% identity) to sequences deposited as *Beauveria bassiana*.

The sequences of *Beauveria bassiana* strain PPRI 5339 was then compared with that of two competitor isolates *viz.* Botanigard® (*B. bassiana* isolate GHA, accession number: JN379811.1) and Naturalis® (*B. bassiana* isolate ATCC 74040, accession number: FJ972972.1) The sequences of the three isolates were found to be 98-99% similar to each other and can be considered near identical species.

MA 1.3.4 Indigenous or non-indigenous at the species level to the intended area of application

Strain PPRI 5339 is not originally indigenous to Europe. It was originally isolated by Dr Schalk Schoeman from the larva of a tortoise beetle, *Conchyloctenia punctata* (Coleoptera: Cassidinae) collected in Escombe, Queensburgh, KwaZulu Natal, South Africa. However, *Beauveria bassiana* is now naturally found across Europe (Rehner, SA, 2005) (Doc M-MA 1.3.4/01).

MA 1.3.5 For mutant or genetically-modified strains, indicate all known differences between the modified micro-organism and the parent wild strain(s)

Strain PPRI 5339 is a naturally occurring strain and has not been genetically altered in any way.

MA 1.3.6 Include any trade names, common names, developmental code names

Trade names: Broadband (South Africa)

The active substance is also sold in different formulations under trade names Broadband EC, Bb Plus and Bb Weevil.

Common names: *Beauveria bassiana* strain PPRI 5339

Developmental code names: Legacy-Becker Underwood development code number for EUP: BUEXP1778
BASF code number for active ingredient: BAS 480 I
BASF code number for EUP: BAS 480 00 I

MA 1.3.7 Relationship to known pathogens

A literature search was conducted by the notifier and no reference to the occurrence of pathogenicity to plants, other animals or humans in closely related species was found.

MA 1.4 Specification of the Material Used for Manufacturing of Formulated Products**MA 1.4.1 Content of the micro-organism**

Please refer to confidential information submitted in Document J.

MA 1.4.2 Identity and content of impurities, additives, contaminating micro-organisms

MA 1.4.2/01

Report: Whittaker, M (2014), Detection and enumeration of *Beauveria bassiana* and microbial contaminants in five production batches of PPRI 5339 technical grade active ingredient. APIS, Knaresborough Technology Park, Manse Lane, Knaresborough, North Yorkshire HG5 8LF. Study Number APIS-BASF-003.

Guidelines: OECD ENV/MC/CHEM(98)17.

GLP: Yes

Summary

This study was conducted to determine the concentration of the microbial pest control agent (MPCA) in five batches of the test item, *Beauveria bassiana* strain PPRI 5339. The mean enumeration result was 1.2×10^{12} cfu/g MPCA. There was no contamination by *Staphylococcus aureus*, *Escherichia coli* or other coliforms, *Salmonella*, *Vibrio cholerae*, *Shigella* or anaerobic bacteria.

Material and Methods

Test Item

Reference Name:	<i>Beauveria bassiana</i> PPRI 5339
Label Identification:	S826
Label Identification:	S827
Label Identification:	S839
Label Identification:	S840
Label Identification:	S840
Nominal purity:	1 x 10 ¹¹ CFU/mL
Stability:	Stable at room temperature or under refrigeration

Active substance enumeration

For each batch, 0.2 g of test item was weighed out to 1 mg in a weighing boat. A paste was formed by gently stirring Tween 80 into the powder with a glass rod. The paste was washed into a sterile Nalgene pot with 100 ml of sterile PBS and sonicated in an ultrasonic bath for 3 minutes. After swirling, the suspension was sonicated for another 3 minutes to reduce clumps. Serial dilutions were prepared in a logarithmic progression by pipetting 1.0 mL of the suspension into 9.0 ml of sterile PBS.

100 µl from the 1 x 10⁻⁷, 1 x 10⁻⁸ and 1 x 10⁻⁹ dilutions was plated in triplicate on Rose Bengal Agar and incubated upright at 28 ± 2 °C for 96 hours. The concentration of active MPCA in each batch of test item was calculated from plate counts as CFU/g. The triplicate plate count from a dilution producing ~30-300 colonies/plate was averaged for each batch and the mean of this calculation for all batches was used to determine an average count for all 5 batches cumulatively. Standard and relative standard deviations were also calculated.

Contaminant screen

26 g of each batch was weighed out accurately (to 1.0 mg) and washed into a sterile flask with 234 mL of sterile phosphate buffered saline (PBS) containing 0.5% Tween 80. This suspension was the stock used for pathogen screening.

1. *Staphylococcus aureus*

1.0 mL of the initial pathogen screening suspension was inoculated onto the surface of each of nine Mannitol Salt agar plates and spread as quickly as possible using a sterile plate spreader. A further three plates were inoculated with a reference culture of *Staphylococcus aureus* to act as a positive control. The plates were allowed to dry with lids on for about 15 minutes at laboratory temperature before being inverted and incubated at 37 ± 2 °C for 24 ± 2 hours. After this time plates were removed and assessed for growth, with typical colonies being yellow on a yellow background and atypical colonies being pink on a pink background.

2. *Escherichia coli* and other coliforms

The presence of *E. coli* was assessed by plating 1.0 mL of the stock suspension onto each of nine TBX plates, prepared according to the manufacturer's instructions. A further three plates were inoculated with a reference culture of *E. coli* to act as a positive control. The plates were allowed to dry with lids on for about 15 minutes at laboratory temperature before being inverted and incubated at 37 ± 2 °C for 48 hours. After this time plates were removed and assessed for growth, with positive colonies being blue-green.

For other coliforms, 1.0 mL of the stock suspension was plated onto the selective and differential media HiChrome Coliform Agar, prepared according to the manufacturer's instructions. A further three plates were inoculated with a reference culture of *E. coli* to act as a positive control. The plates were allowed to dry with lids on for about 15 minutes at laboratory temperature before being inverted and incubated at 37 ± 2 °C for 48 hours. After this time plates were removed and assessed for growth, with *E. coli* forming dark blue/violet colonies and other coliforms forming salmon/red colonies.

3. *Salmonella*

Two approaches were used to detect *Salmonella* contamination. In the first, 1.0 mL of the initial suspension was inoculated onto the surface of each of nine SSA plates and spread as quickly as possible over the surface using a sterile plate spreader. A further three plates were inoculated with a reference culture of *Salmonella typhimurium* to act as a positive control. The plates were allowed to dry with lids on for about 15 minutes at laboratory temperature before being inverted and incubated at 37 ± 2 °C for 48 hours. After this time plates were removed and assessed for growth, with typical colonies being colourless with black centres.

In the second approach, 1.0 ml of the initial suspension was inoculated onto the surface of each of nine HEA plates and spread as quickly as possible over the surface using a sterile plate spreader. A further three plates were inoculated with a reference culture of *Salmonella typhimurium* to act as a positive control. The plates were allowed to dry with lids on for about 15 minutes at laboratory temperature before being inverted and incubated at 37 ± 2 °C for 24 hours. After this time plates were removed and assessed for growth, with typical colonies being blue-green, sometimes with black centres.

4. *Vibrio*

The flasks of stock solution were incubated at 35 ± 2 °C for 7 ± 1 hours, after which a 3 mm loop from the surface pellicle of the culture was transferred to the surface of nine dried HiChrome Vibrio agar plates and streaked in a manner that would yield isolated colonies. No positive control was included due to inherent health risk to lab personnel. Plates were incubated for 18 - 24 hours at 35 ± 2 °C. After this time plates were removed and assessed for growth, with typical colonies being blue-green to purple.

5. *Shigella*

25 g of test item was weighed into 225 ml of *Shigella* broth, to which 0.5 µg/ mL novobiocin had been added following autoclaving. The suspension was held for 10 ± 2 minutes at room temperature and shaken periodically. The supernatant was poured into a sterile 500 mL Erlenmeyer flask and the pH adjusted to 7.0 ± 0.2 with sterile 1 N NaOH.

The flasks were placed in a gas bag containing an AnaeroGen sachet to provide an anaerobic atmosphere and incubated at 44 ± 2 °C for 20 ± 2 hours, after which the enrichment culture suspension was agitated and streaked onto nine SSA and nine HEA plates. No positive control was included due to inherent health risk to lab personnel. Plates were incubated for 20 ± 2 hours at 35 ± 2 °C. After this time plates were removed and assessed for growth, with typical colonies being transparent on both media.

6. Anaerobic bacteria

1.0 mL of the initial suspension was inoculated onto the surface of each of nine TSA plates and spread as quickly as possible over the surface using a sterile plate spreader. A further three plates were inoculated with a reference culture of the obligate anaerobe *Bacteroides fragilis* in thioglycollate broth culture to act as a positive control. The plates were immediately transferred to a gas bag containing an AnaeroGen sachet to provide an anaerobic atmosphere and incubated at 28 ± 2 °C for 72 hours.

Results

Enumeration

The results of the enumeration analysis tests are shown in Table 1.4.2/01-1. The mean result was 1.2×10^{12} CFU/g MPCA germination.

Table 1.4.2/01-1: Enumeration test results for five production batches of *Beauveria bassiana* PPRI 5339 technical grade (viable plate count method)

Batch No	Plate Number			Mean	SEM	Dilution	Vol (mL)	CFU/g
	1	2	3					
S826	102	86	115	101.00	8.39	1×10^{-9}	0.1	1.0×10^{12}
S827	161	160	143	154.67	5.84	1×10^{-9}	0.1	1.5×10^{12}
S839	139	94	141	124.67	15.34	1×10^{-9}	0.1	1.2×10^{12}
S840	75	199	95	123.00	38.44	1×10^{-9}	0.1	1.2×10^{12}
S841	97	128	87	104.00	12.34	1×10^{-9}	0.1	1.0×10^{12}
							Mean CFU/g:	1.2×10^{12}
							SEM:	9.6×10^{10}
							Relative SEM:	17.65

Contamination screen

No contamination by *Staphylococcus aureus*, *Escherichia coli* or other coliforms, *Salmonella*, *Vibrio cholerae*, *Shigella* or anaerobic bacteria was detected in any of the batches tested.

Conclusions

An analysis of five batches of the microbial pest control agent *Beauveria bassiana* strain PPRI 5339 determined the mean active substance concentration to be 1.2×10^{12} CFU/g. A screen for pathogens detected no contamination from *Staphylococcus aureus*, *Escherichia coli* or other coliforms, *Salmonella*, *Vibrio cholerae*, *Shigella* or anaerobic bacteria in any batch.

MA 1.4.3 Analytical profile of batches

Five batch analysis

MA 1.4.3/01

Report: Cheng, KM (2013), Assay to determine Beauvericin within *Beauveria bassiana* concentrate with associated validation. Oxford Analytical Ltd, A3, Telford Road, Bicester, Oxfordshire OX26 4LD, UK. Study Number OA02266.

Guidelines: OECD ENV/MC/CHEM(98)17. 1998

GLP: Yes

Materials and methods

Approximately 1000 mg of sample was accurately weighed into a 50 ml volumetric flask, made to volume with acetonitrile and sonicated for 5 minutes. The samples was allowed to cool and then centrifuged at 3000 rpm for 5 minutes. The sample was then filtered through a Nylon 0.45µm syringe filter. The resulting solution was analysed by LC/MS. Eight ions were monitored using an MSD detector. Samples were quantified against a primary standard of Beauvericin.

Results

The method was demonstrated to be valid for determination of Beauvericin, with linearity, repeatability and recoveries within required limits. The limit of detection for the method was 0.1 mg/kg, with the limit of quantification 0.5 mg/kg.

The results of the assay for of Beauvericin in each batch of *Beauveria bassiana* spore concentrate are shown in Table 1.4.3/01-1.

Table 1.4.3/01-1: Results of the assay for Beauvericin in *Beauveria bassiana* spore concentrates

Batch	Appearance	Impurity content (mg/kg)
BB DC66	The five samples of spore concentrate (powder) were off white in colour (most similar to 10Y9/1 on page R-Y in the Munsell book of colour) with a free-flowing consistency. No clumping or compaction was observed.	Sample A: <LOQ Sample B: <LOQ Mean: <LOQ*
BB DC67		Sample A: <LOQ Sample B: <LOQ Mean: <LOQ*
BB DC68		Sample A: <LOQ Sample B: <LOQ Mean: <LOQ*
BB DC69		Sample A: <LOQ Sample B: <LOQ Mean: <LOQ**
BB DC74		Sample A: <LOQ Sample B: <LOQ Mean: <LOQ*

* The level of *Beauvericin* detected in the samples was less than the 0.5 mg/kg LOQ. The results were detectable at around 0.05 mg/kg but interferences at this level make it difficult to accurately quantify these values.

Conclusions

An analysis of 5 batches of *Beauveria Bassiana* spore concentrate for Beauvericin demonstrated that Beauvericin was not present in the samples above the limit of quantification of 0.5 mg/kg.

In addition, the beauvericin content of an another five production batches of BAS 480 I by analytical method OA02266 with minor modification (refer to MCA 4.1/03).

The results of the analysis are given in Table 1.4.3-1 below.

Table 1.4.3/01-2: Results of the analysis of five production batches of BAS 480 I for the presence of Beauvericin

Batch Number	Concentration of Beauvericin (mg/kg)	Comments
S826	< 0.1	Mean result from duplicate determinations
S827	< 0.1	
S839	< 0.1	
S840	< 0.1	
S841	< 0.1	

The limit of detection of 0.1 mg/kg reported for the latest set of analyses has not been shown to be fully validated. Therefore, a limit of detection of 0.5 mg/kg Beauvericin has been taken forward for risk assessments in this submission.

MA 1.4.3/02

Report: Whittaker, M (2014), Detection and enumeration of yeasts and moulds in five production batches of PPRI 5339 technical grade active ingredient. APIS, Knaresborough Technology Park, Manse Lane, Knaresborough, North Yorkshire HG5 8LF. Report: APIS-BASF-009. Report Date: TBC

Guidelines: OECD ENV/MC/MONO(2011)43

GLP: Yes

Summary

The objective of this study was to determine the yeasts and moulds content in five production batches of *Beauveria bassiana* PPRI 5339 technical grade active ingredient. Yeast and mould contamination was not detected in any batches.

Materials and methods

Approximately 1g of each batch was weighed out accurately and added to 99 ml of sterile (autoclaved) phosphate buffered saline. After vigorous shaking, 1ml of the suspension was plated onto each of nine Yeast and Mould agar plates. Positive controls were prepared by inoculating three plates with a reference culture of *Candida albicans* ATCC 10231 and a further three plates with a reference culture of *Aspergillus brasiliensis* ATCC 16404. Plates were incubated at 26 °C and read after approximately 64 hours.

Results

The results from the yeasts and moulds screens are summarised in Table 1.4.3/02-1. For yeasts and moulds the limit is $<1 \times 10^3$ CFU/g or ml. No growth other than the test item was detected for any of the batches tested.

Table 1.4.3/02-1: Results of the yeasts and moulds screen on 5 production batches of *Beauveria bassiana* PPRI 5339

Batch No.	Plate Number								
	1	2	3	4	5	6	7	8	9
S826	0	0	0	0	0	0	0	0	0
S827	0	0	0	0	0	0	0	0	0
S839	0	0	0	0	0	0	0	0	0
S840	0	0	0	0	0	0	0	0	0
S841	0	0	0	0	0	0	0	0	0
<i>C. albicans</i> ATCC 10231	Good growth	Good growth	Good growth	N/A	N/A	N/A	N/A	N/A	N/A
<i>A. brasiliensis</i> ATCC 16404	Good growth	Good growth	Good growth	N/A	N/A	N/A	N/A	N/A	N/A

Conclusions

An analysis of five production batches of *Beauveria bassiana* PPRI 5339 detected no contamination by yeast and moulds in any batch.

***Beauveria bassiana*
strain PPRI 5339**

DOCUMENT M-MA, Section 2

**BIOLOGICAL PROPERTIES OF THE MICRO-
ORGANISM**

Version

Date	Data points containing amendments or additions and brief description	Document identifier and version number

¹ It is suggested that applicants adopt a similar approach to showing revisions and version history as outlined in SANCO/10180/2013 Chapter 4 How to revise an Assessment Report

Table of Contents

MA 2	BIOLOGICAL PROPERTIES OF THE MICRO-ORGANISM.....	4
MA 2.1	History of the Micro-Organism and its Uses. Natural Occurrence and Geographical Distribution	4
MA 2.1.1	Historical background	4
MA 2.1.2	Origin and natural occurrence	4
MA 2.2	Information on Target Organism	5
MA 2.2.1	Description of target organism	5
MA 2.2.2	Mode of action.....	6
MA 2.2.3	Host Specificity Range and Effects on Species Other Than the Target Harmful Organism.....	7
MA 2.3	Development Stages/Life Cycle of the Micro-Organism	7
MA 2.4	Infectiveness, Dispersal and Colonisation Ability	8
MA 2.5	Relationships to Known Plant or Animal or Human Pathogens.....	9
MA 2.6	Genetic Stability and Factors Affecting It.....	9
MA 2.7	Information on the Production of Metabolites (especially Toxins)	9
MA 2.8	Antibiotics and Other Anti-Microbial Agents	15

MA 2 BIOLOGICAL PROPERTIES OF THE MICRO-ORGANISM

MA 2.1 History of the Micro-Organism and its Uses. Natural Occurrence and Geographical Distribution

The following documents have been used as reference documents for this submission: Zimmermann (2007) (Doc K-MA 2.1/01), and the Draft Assessment Report for *Beauveria bassiana* strain ATCC 74040¹, EFSA Journal 2013;11(4):3612² and EFSA Supporting Publication 2013:EN-518³.

MA 2.1.1 Historical background

There are over 400 reported strains of *Beauveria bassiana* and a few have been commercialised. Most species and strains have some insecticidal properties.

MA 2.1.2 Origin and natural occurrence

PPRI 5339 is a naturally occurring, non-modified strain of *Beauveria bassiana*, originally isolated by Dr Schalk Schoeman from the larva of a tortoise beetle, *Conchyloctenia punctata* (Coleoptera: Cassidinae) collected in Escombe, Queensburgh, KwaZulu Natal, South Africa.

Beauveria bassiana, a hyphomycetous entomopathogenic fungus, is the most widely distributed species of the genus. Domsch *et al.* (1980) (Doc K-MA 2.1.2/01) listed the occurrence and distribution of *B. bassiana* in various countries and habitats. This fungus is generally found throughout a wide range of habitats from alpine soils to heathland, in peat bogs, soils with savannah type vegetation, and in forest and cultivated soils, in sand blows and dunes as well as in desert soils and running water, on all continents of the world.

Natural occurrence of *B. bassiana* was assessed in hedgerow vegetation in Denmark (Meyling & Eilenberg, 2006) (Doc K-MA 2.1.2/02). Populations varied between different plants (*Crataegus monogyna*, *Urtica dioica*, and various members of the *Poaceae*) and between different seasons and were found to be within 0.02 CFU/ and 0.75 CFU/. In general, lower leaves of *U. dioica* had the highest populations (except for July, when upper leaves had the highest populations), and grasses the lowest. Spores are generally considered to originate from the soil. Possible ways of dispersion are rain splashes, especially for lower leaves and grasses, but also dispersion by wind, or by infected or uninfected insects. Cadavers of diseased insects in the phyllosphere are another important source of inoculum.

Beauveria bassiana can grow as saprophyte on simple carbohydrates (e.g. dextrose) and on standard media used in mycological laboratories.

¹ Draft Assessment Report (DAR). Initial risk assessment provided by the rapporteur Member State Germany for the existing active substance *Beauveria bassiana* ATCC 74040, August 2008.

² Scientific opinion on the risks to plant health posed by *Bemisia tabaci* species complex and viruses it transmits for the EU territory, EFSA Journal 2013;11(4):3162 [302pp.]

³ Mudgale, S, De Toni, A, Tostivint, C, Hokkanen, H, Chandler, D (2013) Scientific support, literature review and data collection and analysis for risk assessment on microbial organisms used as active substance in plant protection products – Lot 1 environmental risk characterisation. EFSA Supporting Publication 2013:EN-518

Based on worldwide data, Li (1988) listed 707 insect species as hosts of *B. bassiana*. This list comprised 521 genera and 149 families in 15 orders. In addition, 13 host species of Acarina distributed in 7 genera and 6 families are listed. Nevertheless, despite the prevalence of *B. bassiana* on a huge number of arthropods, it is known that most isolates have a restricted host range (Goettel *et al.*, 1990, Vestergaard *et al.*, 2003) (Doc K-MA 2.1.2/03 and Doc K-MA 2.1.2/04).

Beauveria bassiana has also been isolated from the surface and the interior of plants. It was isolated from bark of elm trees and *Carpinus caroliniana* (Doberski & Tribe 1980; Bills & Polishook, 1991) (Doc K-MA 2.1.2/05 and Doc K-MA 2.1.2/06). Wagner & Lewis (2000) (Doc K-MA 2.1.2/07) and Posada & Vega (2005) (Doc K-MA 2.1.2/08) described the occurrence of *B. bassiana* as endophyte. Airaudi & Marchisio (1996) (Doc K-MA 2.1.2/09) have isolated *B. bassiana* from the air of Turin, Italy.

MA 2.2 Information on Target Organism

MA 2.2.1 Description of target organism

Beauveria bassiana acts as an insecticide, with the primary target being whiteflies (Aleyrodidae), thrips (*Thysanoptera*) and aphids (*Aphididae*). The target whitefly species for this submission are *Bemisia tabaci*, *Trialeurodes vaporariorum* and *Bemisia argentifolii*. The target thrips species for this submission are *Frankliniella occidentalis* and *Thrips tabaci*.

Bemisia tabaci is considered to be one of the most serious threats to crop cultivation worldwide, predominantly because of the large number of viruses it transmits (Virus species transmitted by *B. tabaci* belong to the general Begomovirus, Crinivirus, Ipomovirus, Carlavirus and Torradovirus). In regions where *B. tabaci* is established, viruses transmitted by this insect, especially those affecting tomato and cucurbits, and also beans, pepper and aubergines, are responsible for severe diseases that have a strong negative impact on crop yield. As a consequence, crop production in those areas is not possible without a system-wide and comprehensive set of pest and disease management measures in place. In the risk assessment area, *B. tabaci* is present outdoors in coastal areas with a Mediterranean climate, and in many EU countries the pest is present in greenhouses.

Bemisia tabaci is a complex of at least 28 indistinguishable morphocryptic species, of which four occur in Europe. Two species, Mediterranean (Med, formerly referred to as biotype Q) and Middle East-Asia Minor 1 (MEAM1, formerly referred to as biotype B), are mostly associated with negative effects on crops such as ornamentals, tomato and cucurbits, and are prevalent both in Europe and in many regions across the world (EFSA Journal 11(4): 3162). Independent studies carried out by FERA (UK) have tested the efficacy of Broadband against eggs, instar and adult lifestages of both the B (MEAM1) and Q (Med) biotypes. The product was shown to control both biotypes (Cuthbertson and Brown, 2011) (Doc K-MA 2.2.1/01).

Bemisia tabaci is a small insect, about 1mm long for females and 0.8 mm for males, with a white to light yellow body covered in a waxy powdery material. *Bemisia tabaci* is arrhenotokous: unfertilised eggs give rise to haploid males while fertilised eggs develop into diploid females. *B. tabaci* adults develop from eggs, after passing through four nymphal instars. White eggs, which gradually turn brown, are usually laid in semi-circular arrangement on the underside of leaves. Once hatched, the first nymphal instars, or 'crawlers' are capable of only limited displacement and usually move a few centimetres in search of a feeding site. They initiate feeding on the lower

surface of leaves and moult through the second and third nymphal instar to the fourth, which stops feeding. The nymphs secrete a waxy material at the margins of their body that helps them to adhere to the leaf surface. Development can occur at temperatures between 10°C and 35°C, with an optimum between 25°C and 27°C. Depending on the prevailing temperatures, development from egg to adult lasts between 15 and 70 days. The lifespan of an adult can extend up to 105 days. *Bemisia tabaci* is multivoltine (more than one generation per year), and a female can lay an average of 80 eggs during her lifetime (EFSA Journal 11(4): 3162).

Host range: *B. tabaci* is considered to be a polyphagous insect with a continuously expanding list of host plants, currently comprising more than 1000 plant species. Of the six hundred host plant species described for *B. tabaci* by Oliveira (2001) (Doc K-MA 2.2.1/02) 50% belonged to five families (Fabaceae, Asteraceae, Malvaceae, Solanaceae and Europhobiaceae), comprising a large number of cultivated and non-cultivated annual and perennial plants, including economically important plants (i.e. beans, tomato, cucurbits, poinsettia and many more).

The western flower thrips [*Frankliniella occidentalis* (Pergande)] is an important pest insect in agriculture. This species of thrips is native to the Southwestern United States but has spread to other continents, including Europe, Australia (where it was identified in May 1993), and South America via transport of infested plant material. It has been documented to feed on over 500 different species of host plants, including a large number of fruit, vegetable, and ornamental crops.

The adult male is about 1 mm long; the female is slightly larger, about 1.4 mm in length. Most western flower thrips are female and reproduce by arrhenotokous parthenogenesis; i.e. females can produce males from unfertilized eggs, but females arise only from fertilized eggs. Males are rare, and are always pale yellow, while females vary in color, often by season, from red to yellow to dark brown. Each adult is elongated and thin, with two pairs of long wings. The eggs are oval or kidney-shaped, white, and about 0.2 mm long. The nymph is yellowish in color with red eyes.

The lifecycle of the western flower thrips varies in length due to temperature, with the adult living from two to five or more weeks, and the nymph stage lasting from five to 20 days. Each female may lay 40 to over 100 eggs in the tissues of the plant, often in the flower, but also in the fruit or foliage. The newly hatched nymph feeds on the plant for two of its instars, then falls off the plant to complete its other two instar stages. The insect damages the plant in several ways. The major damage is caused by the adult ovipositing in the plant tissue. The plant is also injured by feeding, which leaves holes and areas of silvery discoloration when the plant reacts to the insect's saliva. Nymphs feed heavily on new fruit just beginning to develop from the flower. The western flower thrips is also the major vector of tomato spotted wilt virus, a serious plant disease.

MA 2.2.2 Mode of action

Like other entomopathogenic fungi, *B. bassiana* attacks its host insects generally percutaneous. The conidia of *B. bassiana* adhere to the insect cuticle by means of hydrophobic interaction between the spore wall and epicuticle lipids. A hydrophobin-type protein and certain enzymes assist in the attachment process. Germination of the conidia and the subsequent successful infection depend on a number of factors, e.g. susceptibility of the host and host stage, and certain environmental factors, such as optimal temperature and humidity. Before penetration, germ tubes may form so-called appressoria and infection pegs. The penetration process is by mechanical means and by the production of several enzymes, including proteases, chitinases and lipases, which degrade the insect cuticle. The penetration is followed by the invasion, which is accompanied by several host immune response activities. During the infection process, *Beauveria*

spp. produces proteolytic enzymes and toxins, while the host insect responds with cellular and humoral defence reactions. In the insect body, the fungus multiplies as blastospores, or yeast-like cells, which are distributed passively in the haemolymph. Enzymes begin to destroy the internal structures of the host insect causing morbidity within 36 to 72 hours. Reduced feeding and immobility are rapidly evident, and the insect dies within 4 to 10 days post-infection. The time to death will depend on the insect species, age and conidial dose. After death of the insect, the fungus starts its saprophytic growth: blastospores transform into mycelia, which emerge through the cuticle. Aerial conidia are formed on the surface of the insect cadaver, which build the characteristic white mould. Sporulation occurs only in conditions of high humidity.

MA 2.2.3 Host Specificity Range and Effects on Species Other Than the Target Harmful Organism

Beauveria bassiana is not host-specific but an opportunistic entomopathogen capable of attacking insects of a wide range of different taxa (Rehner & Buckley 2005, see MA 1.3.1).

Despite the prevalence of *B. bassiana* on a huge number of arthropods, it is known that most isolates have a restricted host range (Goettel *et al.*, 1990, Vestergaard *et al.* 2003). (Doc K-MA 2.2.3/02 and Doc K-MA 2.2.3/03) *Beauveria bassiana* strain PPRI 5339 acts as an insecticide, and the primary target insects are whiteflies (*Aleurodidae*), thrips (*Thysanoptera*).

Based on the wide host range, beneficial insects could be affected by *B. bassiana*. Nevertheless, Hajek and Butler (2000) (Doc K-MA 2.2.3/04) differentiate between the physiological and ecological host range, which means that non-target insects, which are infected under laboratory conditions, may not necessarily be infected in nature (confirmed by Vestergaard *et al.*, 2003) (Doc K-MA 2.2.3/03).

Wang *et al.* (2004) (Doc K-MA 2.2.3/05) monitored the fate of *B. bassiana* strains, which had been inundatively applied against *Dendrolimus punctatus* in Southwest China. They could re-isolate the indigenous and exotic strains during one year. However, the indigenous strains were predominant in the local environment and were not displaced by the exotic ones (Wang *et al.*, 2004) (Doc K-MA 2.2.3/05). An antagonistic effect of *B. bassiana* on plant pathogenic fungi has also been mentioned (Ownley *et al.*, 2004) (Doc K-MA 2.2.3/06).

MA 2.3 Development Stages/Life Cycle of the Micro-Organism

The conidia of *Beauveria bassiana* adhere to the insect cuticle, germinate and penetrate in the insect body, where they replicate as yeast-like cells (blastospores) and destroy the internal structures, causing morbidity within 36 - 72 hours. After death of the insect, the fungus starts its saprophytic growth: blastospores transform into mycelia, which emerge through the cuticle. Aerial conidia are formed on the surface of the insect cadaver, which build the characteristic white growth. *Beauveria bassiana* strain PPRI 5539 requires contact with viable host in order to proliferate.

Beauveria bassiana strain PPRI 5339 has no known sexual stage.

MA 2.4 Infectiveness, Dispersal and Colonisation Ability

Temperature influences the germination, growth and viability of the fungus. In general, *B. bassiana* grows in a wide temperature range from 5 to 35 °C. The optimal growth temperature for *B. bassiana* is 23 to 28 °C, the minimum 5-10 °C and the maximum between 30-35 °C (Fargues *et al.*, 1997; Hallsworth & Magan, 1999) (Doc K-MA 2.4/01 and Doc K-MA 2.4/02). The effect of temperature on growth of *Beauveria bassiana* strain PPRI 5339 has been investigated and is summarized below.

CA 2.4/03

Report: Neethling, D, *et al.* (2012), The effect of temperature on growth of *Beauveria bassiana* strain PPRI 5339. Becker Underwood, Mahogany Ridge, South. Report Date: 21.11.12

Guidelines: None

GLP: No

Summary

The aim of the study was to determine the temperature growth curve of *Beauveria bassiana* strain PPRI 5339. The optimal temperature for growth was determined to be 22 – 28 °C.

Method

A spore suspension of *Beauveria bassiana* strain PPRI 5339 (batch BB 5339 sub3 12072010) was prepared by aseptically removing one loop full of spores from a culture slant and added to 9 ml of sterile saline solution and sonicated for two minutes. The suspension was "stab inoculated" onto 21 Potato Dextrose Agar plates at three points. These three points would develop into three colonies. The plates were incubated at 10°C, 18°C, 22°C, 25°C, 28°C, 32°C and 36°C. Three inoculated plates for each fungus were incubated in each of the seven incubators for 8 days, each of these three plates was considered a replicate. The diameter of the colonies were recorded on days 2, 4 and 8.

Results

After two days there was no growth of the fungal spores at any test temperature. After four days there was growth at temperatures between 22 and 28 °C (Table 2.4-1, Figure 2.4-1). After 8 days growth had also occurred at lower temperatures of 18 °C. There was no growth at either 10, 32 or 36 °C during the 8 day experiment.

Table 2.4-1: Effect of temperature on growth of *Beauveria bassiana* strain PPRI 5339 after 4 days.

Temperature (°C)	Area ()	Standard Deviation
10	0	0.00
18	0	0.00
22	14.74803	1.83
25	34.90659	0.26
28	22.34021	1.83
32	0	0.00
36	0	0.00

Conclusions

Optimal growth of *Beauveria bassiana* strain PPRI 5339 was found to occur at temperatures between 22 and 28 °C.

MA 2.5 Relationships to Known Plant or Animal or Human Pathogens

Endophytic colonisation by *B. bassiana* after spray application was observed in corn, without any signs of pathogenicity (Wagner and Lewis, 2000) (Doc K-MA 2.5/01).

Infection mechanisms of *B. bassiana* are highly evolved and specific only to insects

A literature search conducted by the notifier identified reports of human infestations in immunosuppressed patients but all were successfully treated (Refer to MA 5.1.4).

MA 2.6 Genetic Stability and Factors Affecting It

Fungi possess low genome plasticity due to the narrower scope of mechanisms available to eukaryotic cells for the incorporation of DNA (Coehlo *et al.*, 2013) (Doc K-MA 2.6/01). In line with this, Horizontal Gene Transfer (HGT) is still considered to be anecdotal in fungi (Rosewich and Kistler, 2000) (Doc K-MA 2.6/02) and to date has not been shown to occur in *Beauveria bassiana* (EFSA).

MA 2.7 Information on the Production of Metabolites (especially Toxins)

Information on the metabolites of *Beauveria bassiana* has been taken from the Draft Assessment Report for *Beauveria bassiana* strain ATCC 74040 (Germany, 2008).

Different secondary metabolites isolated from different strains within the genus *Beauveria* have been described in the literature (Strasser *et al.*, 2000) (Doc K-MA 2.7/01). Effects of these metabolites on insects, fungi and bacteria were reported, but also effects on plants, mammals and humans were observed in different experimental systems.

Beauveria metabolites can be divided into four different groups:

1. Low molecular weight compounds: oxalic acid.
2. Non-peptide pigments: oosporein, bassianin, and tenellin.
3. Cyclodepsipeptides: beauvericin, bassianolides, beauveriolides, and beauverolides.
4. High molecular weight proteins (with enzymatic activity): bassiacridin, Bclp.

1. Low molecular weight compounds

Oxalic acid (COOH)₂ is produced by some isolates of *B. bassiana* and is a major pathogenicity factor in the interaction of *B. bassiana* with grasshoppers (*Melanoplus sanguinipes*, Orthoptera; Bidochka & Khachatourians, 1991) (Doc K-MA 2.7/02) and ticks (Ixodidae; Kirkland *et al.*, 2005) (Doc K-MA 2.7/03). Oxalic acid can act as proton donor, electron donor, or as chelating agent to form complexes with divalent cations. Thus, it is able to solubilise insect cuticle components and disrupts cuticles directly (Bidochka & Khachatourians, 1991) (Doc K-MA 2.7/02). Production of oxalic acid by *B. bassiana* isolates depends on composition of the growth media. Activity of *B. bassiana* towards ticks is dependent on acidification and increases drastically below pH 4.5. In addition, acidification is required for activity of lytic enzymes from *B. bassiana*, which makes oxalic acid a synergistic facilitator of pathogenesis. Oxalic acid is as well produced by plant pathogenic fungi to penetrate plant cell walls, but also by plants used for human consumption like rhubarb, spinach and beets to prevent insects from feeding. Therefore, oxalic acid is not expected to pose any risk to humans or the environment.

2. Non-peptide pigments

Oosporein, bassianin, and tenellin are red, orange, or yellow pigments produced by *Beauveria* strains. They share similar modes of action.

Jeffs & Khachatourians (1997) (Doc K-MA 2.7/04) analysed the effect of tenellin, bassianin and oosporein on ATPase activity. All three metabolites inhibited total erythrocyte membrane ATPase activity in a dose-dependent manner. The effect was more pronounced for ⁺ATPases than for Na⁺/K⁺-ATPase activity. The ATPase inhibitory activity for these pigments was not specific but was probably a consequence of membrane disruption, since pigments all caused alterations in erythrocyte morphology and promoted varying degrees of cell lysis.

Eley *et al.* (2007) (Doc K-MA 2.7/05) analysed biosynthesis of tenellin and produced a mutant lacking genes encoding tenellin biosynthesis enzymes. This strain was as efficient as the wild type strain towards *G. mellonella* larvae, showing that tenellin is not involved in insect pathogenesis.

Oosporein

Oosporein (or oosporin) is a red-coloured dibenzoquinone which is produced by some strains of the genus *Beauveria*, most of them belonging to the species *B. brogniartii*. Oosporein acts as an antibiotic against gram-positive bacteria. Oosporein produced by the cereal post-harvest pathogen *Chaetomium trilaterale* in poultry feed was found to be toxic to chicken (Pegram & Wyatt, 1981) (Doc K-MA 2.7/06). Oosporein is supposed to inhibit activity of enzymes through redox modification of SH groups and to inhibit membrane ATPase activity.

Oosporein together with other metabolites produced by fungi used for biocontrol was analysed for acute toxicity towards the crustaceae *Artemia salina* and *Daphnia magna*. Among all metabolites tested, oosporein showed the lowest toxicity in these systems and data for oosporein in *A. salina* could not be determined. However, sensitivity of *D. magna* to oosporein increased when exposure was extended from 24 h to 36 h (Favilla *et al.*, 2006) (Doc K-MA 2.7/07).

Oosporein has an antifungal effect on the plant pathogen *Phytophthora infestans* (Nagaoka *et al.* 2004) (Doc K-MA 2.7/08). Strasser *et al.* (2000) (Doc K-MA 2.7/01) demonstrated that production of oosporein by a *B. brogniartii* strain varies between 3.2 mg/kg for solid phase

fermentation on barley kernels and 300 mg/L for liquid media. Accumulation of oosporein and other metabolites involved in insect pathogenicity only occurs during parasitism of insects (Strasser *et al.*, 2000) (Doc K-MA 2.7/01).

Abendstein *et al.* (2000) (Doc K-MA 2.7/09) applied *Beauveria brogniartii* to seed potatoes in a rate 40-fold higher than the recommended application rate. No phytotoxic effect was observed, and *B. brogniartii* did not colonise potato tuber tissue. No oosporein was detected in tubers after harvest of treated plants, nor in tubers spiked with oosporein. This may have resulted from analytical problems in detection or from metabolisation or degradation of oosporein within the tubers. Oosporein was metabolised in potato sap within two hours, indicating that this metabolite is not accumulated by plants.

Bassianin, tenellin, and oosporein are produced by some *B. bassiana* strains during interaction with their target hosts or under defined conditions in liquid media. Contents of bassianin, tenellin, or oosporein were not determined for *Beauveria bassiana* strain PPRI 5339. Accumulation of these compounds in natural environments or after application of plant protection products containing *B. bassiana* was not analysed, but does not seem realistic. Accumulation and subsequent effects on vertebrates were only reported for oosporein produced by *Chaetomium trilaterale*, a saprophytic fungus multiplying in poultry feed. Exposure of humans or non-target organisms to bassianin, tenellin, and oosporein can only occur through consumption of insects infested with *B. bassiana*. However, this exposure already exists as *B. bassiana* is a naturally occurring fungus in Europe.

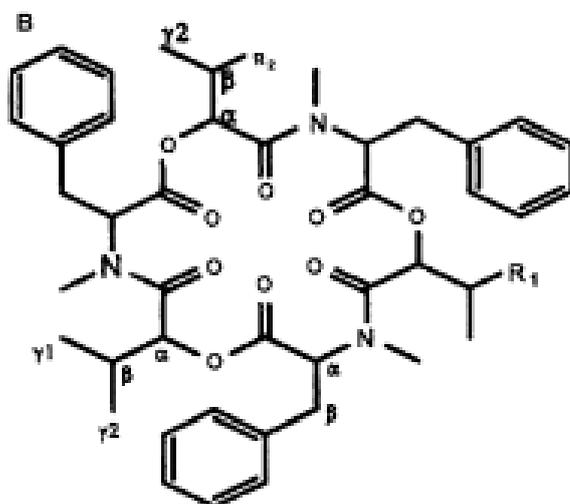
3. Cyclodepsipeptides

This group includes relatively small cyclic peptides as beauvericin, bassianolides, beauveriolides, and beauverolides. They act as ionophore antibiotics and can lead to membrane disruption.

Beauvericin is a cyclic hexadepsipeptide which has been isolated from members of the entomopathogenic fungi genera *Beauveria* and *Paecilomyces*, but also from plant pathogenic or saprophytic fungi of the genus *Fusarium* that are common maize and wheat parasites. Beauvericin has antibiotic activity against gram-positive and gram-negative bacteria and moderate insecticidal activity against certain groups of insects (Strasser *et al.*, 2000) (Doc K-MA 2.7/01). It is toxic towards several shrimp species and has even shown toxicity towards murine and human cell lines (Strasser *et al.*, 2000) (Doc K-MA 2.7/01). Beauvericin forms Na⁺ and K⁺ complexes leading to increased permeability of membranes. Beauvericin leads to cell death in human acute lymphoblastic leukemia cells through induction of an apoptotic pathway (Jow *et al.*, 2004) (Doc K-MA 2.7/10). Champlin and Grula (1979) (Doc K-MA 2.7/11) could not detect beauvericin in spent media of a *B. bassiana* culture or in the haemolymph of *Heliothis zea* larvae infected with the same strain, which shows high activity against *H. zea* larvae. Likewise, direct injection of beauvericin did not affect *H. zea* larvae whereas bassianolide paralyzes *H. zea* larvae. At least for the interaction between this *B. bassiana* strain and *H. zea*, it can be concluded that beauvericin is not a pathogenicity factor. Similar results were reported for *Bombyx mori* larvae. On the other hand, Calo *et al.* (2004) (Doc K-MA 2.7/12) reported cytotoxicity of beauvericin in a *Spodoptera frugiperda* cell culture.

Ten production lots of *Beauveria bassiana* strain PPRI 5339 were analysed for beauvericin contents (Cheng, KM, 2013 submitted in Doc M, Annex Point MA 1.4.3/02) and no beauvericin was detected above the limit of quantification in any of the lots. It can be concluded the *B. bassiana* PPRI 5339 does not accumulate beauvericin to relevant levels during growth on

synthetic media as used during production of BAS 480 I. As summarised above, beauvericin is not necessarily part of insect pathogenesis by *B. bassiana* species.

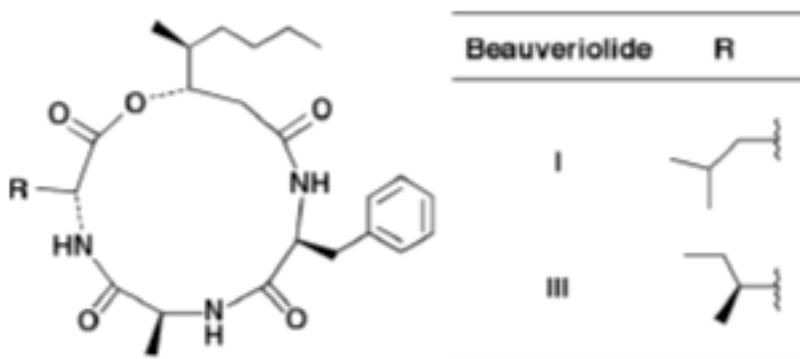


Beauvericin, $R_1 = R_2 = \text{CH}_3$

Chemical structure of beauvericin (Jow *et al.* 2004)

Bassianolide is a cyclo-octadepsipeptide which shows insecticidal activities (Champlin & Grula 1979) (Doc K-MA 2.7/11). Overall, little is known on toxicity of bassianolide towards mammals or plants. Production of bassianolide was not analysed for *B. bassiana* strain PPRI 5339.

Beauveriolides are cyclotetradepsipeptides and consist e.g. of L-Phe, L-Ala, D-Leu and (3S,4S)-3-hydroxy-4-methyloctanoic acid (beauveriolide I), and L-Phe, L-Ala, D-allo-Ile, and 3-hydroxy-4-methyloctanoic acid (beauveriolide III). Beauveriolides I and III can be extracted from the culture broth of a *Beauveria sp.* strain originally isolated from soil. Production depends on the supply of specific amino acids. Beauveriolides I and III inhibit cholesteryl ester synthesis through inhibition of activity of acyl-CoA:cholesterol acyltransferase (ACAT). ACAT is the key enzyme catalysing synthesis of cholesteryl esters that are deposited in lipid droplets of macrophages that lead to atherosclerosis in the arterial wall. No cytotoxic effect was observed.



Structure of beauveriolides (Namatame *et al.* 2004) (Doc K-MA 2.7/13)

Beauverolides are structurally closely related to beauveriolides. They are as well

cyclotetrapeptides and consist of three amino acids and a methyl-octanoyl or methyl-decanoyl moiety. Up to now, 15 different beauverolides have been characterised. Different beauverolides are produced by the same *Beauveria bassiana* strain (Kuzma *et al.* 2001) (Doc K-MA 2.7/14), but also by *Paecilomyces fumosoroseus* species (Jegorov *et al.* 1994) (Doc K-MA 2.7/15). Biological functions of beauverolides are not clear, since they do not possess bactericidal, fungicidal, or direct insecticidal properties. Beauverolide L from *B. brogniartii* does not cause mortality in *Galleria mellonella* larvae, but affects humoral responses. Incubation of isolated plasmatocytes with beauverolide L led to cytoskeleton alterations. A role in insect pathogenicity is possible through exposition of beauverolide on the outer membrane of hyphae penetrating the insect body (Vilcinskas *et al.* 1999) (Doc K-MA 2.7/16). Effects of beauverolides and beauverolides on plants, insects, or mammals are unknown. The unpolar nature of beauverolides (Vilcinskas *et al.* 1999) makes secretion by the fungus outside the infected host highly improbable. Uptake by plants via leaves or roots seems impossible.

4. High molecular weight proteins

Two larger proteins with toxic activity against host insects were described so far, namely Bassiacridin and Bclp.

Bassiacridin was purified from a *Beauveria bassiana* strain originally isolated from the locust *Docioestaurus maroccanus* (Orthoptera; Quesada-Moraga and Vey, 2004) (Doc K-MA 2.7/17). Bassiacridin is a monomer protein with a molecular weight of 60 kDa exhibiting β -glucosidase, β -galactosidase and N-acetylglucosaminidase activities. Melanisation and necrotisation were observed in larvae injected with bassiacridin. Cultured cells from *Spodoptera frugiperda* (Sf-9) were sensitive and revealed reduced metabolic activity and morphological changes. Cells showed hypertrophy, disruption of organelles and cell membranes, and intense vacuolisation. Bassiacridin is the only toxic component in the interaction between this *B. bassiana* strain and locusts, which might either be caused by absence of production of other metabolites or by the capacity of Orthoptera to inactivate cyclic depsipeptides. Upon injection, mortality in nymphs from *Locusta migratoria*, *Docioestaurus maroccanus*, and *Schistocerca gregaria* was observed. Activity is specific for locusts as *Tenebrio molitor* and *Spodoptera littoralis* larvae were not affected. *Galleria mellonella* larvae only showed low toxic effects. Due to the specificity of the effect for Orthoptera hosts, no side-effects on non-target organisms are expected. Production of bassiacridin in other *B. bassiana* strains was not analysed.

Fuguet and Vey (2004) (Doc K-MA 2.7/18) compared mechanisms of mycoses of different *Beauveria* strains (eleven *B. bassiana* strains and five strains from different *Beauveria* species) after injection of spores into *Galleria mellonella* larvae. Symptoms were classified into four groups according to the melanisation of the larvae, showing that pathogenicity mechanisms differ between strains within the species *B. bassiana*, but also among other *Beauveria* species. Mycelium development also differed among strains, with strong mycelium growth associated to the non-melanising type. Two model strains were selected, representing types of pathogenicity linked to melanisation and non-melanisation. Soluble components in the haemolymph of infected larvae were able to cause the same symptoms as living spores. Further characterisation revealed haemolymph components to be thermolabile proteins for the melanising strain and low-molecular weight components for the non-melanising strain. Fuguet *et al.* (2004) (Doc K-MA 2.7/19) used the same melanising model strain to characterise a chitosanase-like protein (Bclp) secreted into the medium *in vitro* that upon injection into *Galleria mellonella* larvae caused the same cytotoxic effects and cuticle alterations as those during infection by *B. bassiana*. The non-melanising strain lacked this protein.

Cephalosporolides

In addition to those metabolites listed above, Oller-Lopez *et al.* (2005) reported the formation of metabolites cephalosporolides, E and F along with another previously unknown chemical structure, referred to as bassianolone. These metabolites were produced under low nitrogen, stress conditions. More recently, however, Song *et al.* (2014), have shown that the structure of bassianolone is identical to cephalosporolide C (Ces-C), a known naturally occurring compound. Ces C is the natural precursor to cephalosporolides E and F, undergoing acid catalyzed dehydrative rearrangement. Oller-Lopez *et al.* (2005), showed that Ces-E and F have no antimicrobial activity, whereas Ces-C (bassianolone) has been shown to have significant antimicrobial activity.

Conclusions

Taken together, *B. bassiana* strains are able to synthesise metabolites with very different chemical and biological properties. Potential effects on humans or the environment also differ between metabolites. Some of these metabolites obviously are key determinants in pathogenicity for *B. bassiana* towards their host insects. Effects of metabolites on target hosts depend on both the *Beauveria* strains that differ in the production of metabolites and the target insects that differ in susceptibility towards metabolites. It has to be pointed out that no detrimental *in vivo* effects of metabolites produced by *B. bassiana* are known so far on humans or non-target organisms. Effects were only described for purified metabolites in cell cultures. Reports on detrimental effects of beauvericin or oosporein on chicken refer to production of this metabolite by *Fusarium spp* or *Chaetomium spp.*, respectively.

This information on *Beauveria* metabolites was summarized for different strains and only reflects biosynthetic capacities within the genus *Beauveria*. Most *Beauveria* strains were only assessed for a single type of secondary metabolites, and no specific information on the capacity to produce different groups of metabolites with potential impact on humans or the environment is available for any strain, including *B. bassiana* strain PPRI 5339. Moreover, conditions under which certain metabolites are produced are widely unknown. For all groups of metabolites, the actual production *in vitro* depends on the media in which isolates are grown and on the age of the culture. Original studies on metabolites were rather focussed on chemical structures and biosynthetic capacities than on the ecological role of the respective metabolites. Accumulation of metabolites was observed either in nutrient rich media (and depends on the composition of the media), or locally during parasitism of the target host (Strasser *et al.*, 2000; Fuguet and Vey, 2004). In addition, one or more of several possible pathways may be enhanced or repressed by environmental conditions.

Beauveria bassiana does not multiply or show metabolic activity in the absence of host insects, and in consequence no accumulation of potentially harmful metabolites to levels that might affect the environment is expected. Low amounts of the right metabolites produced and secreted at the right time and place may be sufficient for biocontrol efficacy.

Stability and accumulation of metabolites under conditions of application of *B. bassiana* products seems to be very limited by high metabolic activity of soil micro-organisms. Therefore, accumulation of metabolites with toxic properties towards man or the environment after application of plant protection products containing *B. bassiana* is highly improbable, and exposure of humans or non-target organisms to metabolites from *B. bassiana* can only occur through consumption of insects infested with *B. bassiana*. However, this exposure does not exist for

humans and any potential exposure for insectivorous animals to metabolites of *B. bassiana* already existed before the use of this microbial active substance as *B. bassiana* is a naturally occurring fungus in Europe.

An additional literature search was carried out by the Rapporteur Member State, Germany in the Draft Assessment Report for *Beauveria bassiana* strain ATCC .

Based on these findings, it can be concluded that *Beauveria bassiana* strain PPRI 5339 poses no risk to man or the environment via the production of toxic metabolites.

MA 2.8 Antibiotics and Other Anti-Microbial Agents

Beauveria bassiana is an opportunistic pathogen that has very rarely caused infections in humans and is not related to any known human pathogen.

Within the open literature, two instances of potential resistance to antifungal agents have been reported. Figuera *et al.* (2012) (Doc K-MA 2.8/01) identified a potential resistance of *Beauveria bassiana* to voriconazole, fluconazole and amphotericin B. Sonoyama *et al.* (2008) (Doc K-MA 2.8/02) also identified resistance to fluconazole and amphotericin B, but identified voriconazole as the treatment of choice. Spread of resistance (genes) to antimicrobial agents between fungal species is not of that concern as for bacteria with regard to antibiotics.

***Beauveria bassiana*
strain PPRI 5339**

DOCUMENT M-MA, Section 3

**FURTHER INFORMATION ON THE MICRO-
ORGANISM**

Version

Date	Data points containing amendments or additions and brief description	Document identifier and version number

¹ It is suggested that applicants adopt a similar approach to showing revisions and version history as outlined in SANCO/10180/2013 Chapter 4 How to revise an Assessment Report

Table of Contents

MA 3	FURTHER INFORMATION ON THE MICRO-ORGANISM	4
MA 3.1	Function	4
MA 3.2	Field of Use Envisaged	4
MA 3.3	Crops or Products Protected or Treated	4
MA 3.4	Method of Production and Quality Control.....	4
MA 3.5	Information on Occurrence or Possible Occurrence of the Development of Resistance of the Target Organism	4
MA 3.5.1	Potential development of resistance to <i>Beauveria bassiana</i> strain PPRI 5339.....	4
MA 3.5.2	Potential development of cross resistance between <i>Beauveria bassiana</i> strain PPRI 5339 and synthetic chemical insecticides.....	5
MA 3.5.3	Potential development of cross resistance between <i>Beauveria bassiana</i> strain PPRI 5339 and other microbial plant protection products.....	5
MA 3.5.4	International guidelines on management of potential resistance.....	6
MA 3.6	Methods to Prevent Loss of Virulence of Seed Stock of the Micro- Organism	6
MA 3.7	Recommended Methods and Precautions Concerning Handling, Storage, Transport or Fire.....	6
MA 3.8	Procedures for Destruction or Decontamination.....	6
MA 3.9	Measures in Case of an Accident.....	6

MA 3 FURTHER INFORMATION ON THE MICRO-ORGANISM

MA 3.1 Function

Contact insecticide.

MA 3.2 Field of Use Envisaged

Protected glasshouse on crops and ornamentals.

MA 3.3 Crops or Products Protected or Treated

Beauveria bassiana strain PPRI 5339 is intended to be used in glasshouse production of fruits and vegetables and ornamentals at a rate of 100 g a.s./ha for protection against thrips (*Frankliniella occidentalis* and *Thrips tabaci*) and whitefly (*Trialeurodes vaporariorum*, *Bemisia tabaci* and *Bemisia argentifolii*). (Refer to Document D-1.)

MA 3.4 Method of Production and Quality Control

All documents relating to the manufacture and quality control of the representative formulation can be found in Confidential Document J.

MA 3.5 Information on Occurrence or Possible Occurrence of the Development of Resistance of the Target Organism

MA 3.5.1 Potential development of resistance to *Beauveria bassiana* strain PPRI 5339

To date there have been no published reports of resistance development in field populations to any product containing the active ingredient *Beauveria bassiana*. There have also been no published studies demonstrating the development of resistance to any strain of *Beauveria bassiana* from a laboratory culture of an economically important pest species of protected horticulture as claimed in the *Beauveria bassiana* strain PPRI 5339 GAP. The limited research on laboratory insect populations has shown potential for development of resistance to *Beauveria bassiana* in *Drosophila melanogaster* (Diptera) and *Galleria mellonella* (Lepidoptera), common standard laboratory test organisms. Under laboratory conditions Dubovskiy *et al.* (2013) (Doc M-MA 3.5.1-01) reported that under constant selective pressure from *B. bassiana*, 25th generation larvae of *Galleria mellonella* exhibited significantly enhanced resistance, which was specific to this pathogen and not to another insect pathogenic fungus *Metarhizium anisopliae*. Dubovskiy *et al.* (2013) hypothesised that the resistance was due to increased prioritisation of pathogen-species-specific defence mechanisms. In this study, systemic immune defences were suppressed in order to allow more resources to be prioritised for enhanced response in the cuticle and epidermis for example the increased expression of the fungal enzyme inhibitor IMPI [insect metalloproteinase inhibitor] and cuticular phenoloxidase activity. Understanding the mode of action of this resistance development demonstrates that, if this development of resistance is also able to occur in wild populations of insects of agricultural importance surrounded by other selection pressures, it will be possible to manage the resistance within a pest population by rotating with other biological control agents e.g. *Metarhizium anisopliae* (Dubovskiy *et al.* (2013) showed the *B. bassiana* resistance mechanism was specific to this species of fungus) or

even potentially different strains of *B. bassiana* which might produce a different range of insect cuticle degrading enzymes not prevented from working by the fungal enzyme inhibitor and cuticular phenoloxidase activity in the resistant population. Dudovskiy et al. (2013) hypothesised that development of resistance to *B. basisana* was a costly trait and rather than being compensated for by a reduction in life-history traits (e.g. fecundity or longevity) the insect would reduce the energy put into other areas of its immune system which would make it more susceptible to other pathogens, which would be to the benefit of an IPM program. Dubovskiy et al., (2013) concluded that, in contrast to insects that develop resistance to synthetic chemical insecticides where a slight change can have a profound effect (e.g. aphid, Silva et al., (2012) (Doc M-MA 3.5.1-02); whitefly, Alon et al. (2008) (Doc M-MA 3.5.1-03); red flour beetle, Arnaud and Haubruge (2002) (Doc M-MA 3.5.1-04)) resistance to *B. bassiana* would involve a multiple array of interdependent traits. There are multiple processes involved in infection with *B. bassiana* which reduces the potential for resistance to develop (Gillespie et al. (2000) (Doc M-MA 3.5.1-05)). In addition Dudovskiy et al. (2013) concluded that increased resistance to a specific strain of *B. bassiana* did not pose a major threat to the use of pathogenic fungi as biocontrol agents due to three factors 1) There is no cross resistance to other fungal species, 2) There is a cost to the development of resistance with reduced fecundity 3) down regulation of other parts of the immune system will probably make the insect more susceptible to other opportunistic microbial pathogens.

In another study to identify potential development of resistance to *B. bassiana* in *Drosophila melanogaster* Kraaijeveld et al. (2012) (Doc M-MA 3.5.1-06) found no evidence for development of resistance to *B. bassiana* after 15 generations of selection suggesting that development of resistance to *B. bassiana* may be either a mechanism that not all species are capable of and/or a complex evolutionary change that requires many generations to develop. Kraaijeveld and Godfray (2008) (Doc M-MA 3.5.1-07) discussed the potential for resistance or tolerance to *B. bassiana* in *Drosophila melanogaster* and concluded that "resistance to fungal biopesticides will not evolve easily".

MA 3.5.2 Potential development of cross resistance between *Beauveria bassiana* strain PPRI 5339 and synthetic chemical insecticides.

Because of the nature of *B. bassiana* strain PPRI 5339 cross-resistance with chemical insecticide active ingredients is unlikely.

MA 3.5.3 Potential development of cross resistance between *Beauveria bassiana* strain PPRI 5339 and other microbial plant protection products.

Following penetration into the insect haemolymph, the insect immune response to both fungal and bacterial microbial pathogens is to produce a range of peptides to kill the infection or encapsulation to prevent it reproducing. However, the immune defence systems of insects have been shown to give different humoral responses upon infection with parasitic fungi such as *Beauveria bassiana* compared to after challenge with bacteria (Wojda et al. (2009) (Doc M-MA 3.5.3-01)) Therefore with different defence pathways, cross-resistance between different kingdoms of microbial pathogens is unlikely.

MA 3.5.4 International guidelines on management of potential resistance.

Insecticide Resistance Action Committee (IRAC) is the industry body that monitors, manages and provides recommendations for the management of resistance to insecticides. Currently no biological active ingredients (such as entomopathogenic fungi *B. bassiana*) are included on their classification of resistance management codes therefore it is not possible to provide an IRAC code for *Beauveria bassiana* strain PPRI 5339. Insecticide Resistance Action Committee does however state that management of potential resistance development to any insecticide should “include effective cultural and biological control practices that work in harmony with effective IPM [Integrated Pest Management] programmes”.

MA 3.6 Methods to Prevent Loss of Virulence of Seed Stock of the Micro-Organism

As part of the production process the strain is passed through an insect (termite) to maintain pathogenicity. Sourcing back from an infected insect rather than sub-culturing at the end of a production batch maintains genetic stability (Refer to confidential information in Document J for further details).

Production is started from a culture maintained within a host insect (termite) rather than sub-sampling commercial production batches which will help maintain genetic stability.

MA 3.7 Recommended Methods and Precautions Concerning Handling, Storage, Transport or Fire

A material safety data sheet for *Beauveria bassiana* PPRI 5339 is included in Document H.

MA 3.8 Procedures for Destruction or Decontamination

Leftover quantities of the product must be disposed of in a commercial incinerator according to local regulations. Incineration must be done under controlled conditions according to the directives 94/67/EC and 2000/76/EC:

- Residence time greater than 2 seconds
- Presence of more than 6 % of oxygen
- Temperature above 850°C

Contaminated packaging has to be thoroughly rinsed with plenty of water, then burned in a commercial incinerator. In field applications the washing solutions have to be added to the spray mixture, otherwise they have to be burned in a commercial incinerator.

MA 3.9 Measures in Case of an Accident

A material safety data sheet for *Beauveria bassiana* PPRI 5339 is included in Document H.

***Beauveria bassiana*
strain PPRI 5339**

DOCUMENT M-MA, Section 4

ANALYTICAL METHODS

Version

Date	Data points containing amendments or additions and brief description	Document identifier and version number

¹ It is suggested that applicants adopt a similar approach to showing revisions and version history as outlined in SANCO/10180/2013 Chapter 4 How to revise an Assessment Report

Table of Contents

MA 4	ANALYTICAL METHODS.....	4
MA 4.1	Methods for the Analysis of the Micro-Organism as Manufactured.....	4
MA 4.1.1	Methods for the determination of relevant metabolites.....	12
MA 4.2	Methods to Determine and Quantify Residues (Viable or Non-Viable)	14

MA 4 ANALYTICAL METHODS

MA 4.1 Methods for the Analysis of the Micro-Organism as Manufactured

MA 4.1/01

Report: Whittaker, M (2014), Detection and enumeration of *Beauveria bassiana* and microbial contaminants in five production batches of PPRI 5339 technical grade active ingredient. APIS, Knaresborough Technology Park, Manse Lane, Knaresborough, North Yorkshire HG5 8LF. Study Number APIS-BASF-003.

Guidelines: OECD ENV/MC/CHEM(98)17.

GLP: Yes

Summary

This study was conducted to determine the concentration of the microbial pest control agent (MPCA) in five batches of the test item, *Beauveria bassiana* strain PPRI 5339. The mean enumeration result was 1.2 x CFU/g MPCA. There was no contamination by *Staphylococcus aureus*, *Escherichia coli* or other coliforms, *Salmonella*, *Vibrio cholerae*, *Shigella* or anaerobic bacteria.

Material and Methods

Test Item

Reference Name: *Beauveria bassiana* PPRI 5339

Label Identification: S826

Label Identification: S827

Label Identification: S839

Label Identification: S840

Label Identification: S840

Nominal purity: 1 x CFU/mL

Stability: Stable at room temperature or under refrigeration

Active substance enumeration

For each batch, 0.2 g of test item was weighed out to 1 mg in a weighing boat. A paste was formed by gently stirring Tween 80 into the powder with a glass rod. The paste was washed into a sterile Nalgene pot with 100 mL of sterile PBS and sonicated in an ultrasonic bath for 3 minutes. After swirling, the suspension was sonicated for another 3 minutes to reduce clumps. Serial dilutions were prepared in a logarithmic progression by pipetting 1.0 mL of the suspension into 9.0 mL of sterile PBS.

100 µl from the 1×10^{-7} , 1×10^{-8} and 1×10^{-9} dilutions was plated in triplicate on Rose Bengal Agar and incubated upright at 28 ± 2 °C for 96 hours. The concentration of active MPCA in each batch of test item was calculated from plate counts as CFU/g. The triplicate plate count from a dilution producing ~30-300 colonies/plate was averaged for each batch and the mean of this calculation for all batches was used to determine an average count for all 5 batches cumulatively. Standard and relative standard deviations were also calculated.

Contaminant screen

26 g of each batch was weighed out accurately (to 1.0 mg) and washed into a sterile flask with 234 mL of sterile phosphate buffered saline (PBS) containing 0.5% Tween 80. This suspension was the stock used for pathogen screening.

1. *Staphylococcus aureus*

1.0 mL of the initial pathogen screening suspension was inoculated onto the surface of each of nine Mannitol Salt agar plates and spread as quickly as possible using a sterile plate spreader. A further three plates were inoculated with a reference culture of *Staphylococcus aureus* to act as a positive control. The plates were allowed to dry with lids on for about 15 minutes at laboratory temperature before being inverted and incubated at 37 ± 2 °C for 24 ± 2 hours. After this time plates were removed and assessed for growth, with typical colonies being yellow on a yellow background and atypical colonies being pink on a pink background.

2. *Escherichia coli* and other coliforms

The presence of *E. coli* was assessed by plating 1.0 mL of the stock suspension onto each of nine TBX plates, prepared according to the manufacturer's instructions. A further three plates were inoculated with a reference culture of *E. coli* to act as a positive control. The plates were allowed to dry with lids on for about 15 minutes at laboratory temperature before being inverted and incubated at 37 ± 2 °C for 48 hours. After this time plates were removed and assessed for growth, with positive colonies being blue-green.

For other coliforms, 1.0 mL of the stock suspension was plated onto the selective and differential media HiChrome Coliform Agar, prepared according to the manufacturer's instructions. A further three plates were inoculated with a reference culture of *E. coli* to act as a positive control. The plates were allowed to dry with lids on for about 15 minutes at laboratory temperature before being inverted and incubated at 37 ± 2 °C for 48 hours. After this time plates were removed and assessed for growth, with *E. coli* forming dark blue/violet colonies and other coliforms forming salmon/red colonies.

3. *Salmonella*

Two approaches were used to detect *Salmonella* contamination. In the first, 1.0 mL of the initial suspension was inoculated onto the surface of each of nine SSA plates and spread as quickly as possible over the surface using a sterile plate spreader. A further three plates were inoculated with a reference culture of *Salmonella typhimurium* to act as a positive control. The plates were allowed to dry with lids on for about 15 minutes at laboratory temperature before being inverted and incubated at 37 ± 2 °C for 48 hours. After this time plates were removed and assessed for growth, with typical colonies being colourless with black centres.

In the second approach, 1.0 mL of the initial suspension was inoculated onto the surface of each of nine HEA plates and spread as quickly as possible over the surface using a sterile plate spreader. A further three plates were inoculated with a reference culture of *Salmonella typhimurium* to act as a positive control. The plates were allowed to dry with lids on for about 15 minutes at laboratory temperature before being inverted and incubated at 37 ± 2 °C for 24 hours. After this time plates were removed and assessed for growth, with typical colonies being blue-green, sometimes with black centres.

4. *Vibrio*

The flasks of stock solution were incubated at 35 ± 2 °C for 7 ± 1 hours, after which a 3 mm loop from the surface pellicle of the culture was transferred to the surface of nine dried HiChrome Vibrio agar plates and streaked in a manner that would yield isolated colonies. No positive control was included due to inherent health risk to lab personnel. Plates were incubated for 18 - 24 hours at 35 ± 2 °C. After this time plates were removed and assessed for growth, with typical colonies being blue-green to purple.

5. *Shigella*

25 g of test item was weighed into 225 mL of *Shigella* broth, to which 0.5 µg/ mL novobiocin had been added following autoclaving. The suspension was held for 10 ± 2 minutes at room temperature and shaken periodically. The supernatant was poured into a sterile 500 mL Erlenmeyer flask and the pH adjusted to 7.0 ± 0.2 with sterile 1 N NaOH.

The flasks were placed in a gas bag containing an AnaeroGen sachet to provide an anaerobic atmosphere and incubated at 44 ± 2 °C for 20 ± 2 hours, after which the enrichment culture suspension was agitated and streaked onto nine SSA and nine HEA plates. No positive control was included due to inherent health risk to lab personnel. Plates were incubated for 20 ± 2 hours at 35 ± 2 °C. After this time plates were removed and assessed for growth, with typical colonies being transparent on both media.

6. Anaerobic bacteria

1.0 mL of the initial suspension was inoculated onto the surface of each of nine TSA plates and spread as quickly as possible over the surface using a sterile plate spreader. A further three plates were inoculated with a reference culture of the obligate anaerobe *Bacteroides fragilis* in thioglycollate broth culture to act as a positive control. The plates were immediately transferred to a gas bag containing an AnaeroGen sachet to provide an anaerobic atmosphere and incubated at 28 ± 2 °C for 72 hours.

Results

Enumeration

The results of the enumeration analysis tests are shown in Table 4.1/01-1. The mean result was 1.2 x CFU/g MPCA germination.

Table 4.1/01-1: Enumeration test results for five production batches of *Beauveria bassiana* PPRI 5339 technical grade (viable plate count method)

Batch No	Plate Number			Mean	SEM	Dilution	Vol (mL)	CFU/g
	1	2	3					
S826	102	86	115	101.00	8.39	1 x 10 ⁻⁹	0.1	1.0 x
S827	161	160	143	154.67	5.84	1 x 10 ⁻⁹	0.1	1.5 x
S839	139	94	141	124.67	15.34	1 x 10 ⁻⁹	0.1	1.2 x
S840	75	199	95	123.00	38.44	1 x 10 ⁻⁹	0.1	1.2 x
S841	97	128	87	104.00	12.34	1 x 10 ⁻⁹	0.1	1.0 x
							Mean CFU/g:	1.2 x
							SEM:	9.6 x
							Relative SEM:	17.65

Contamination screen

No contamination by *Staphylococcus aureus*, *Escherichia coli* or other coliforms, *Salmonella*, *Vibrio cholerae*, *Shigella* or anaerobic bacteria was detected in any of the batches tested.

Conclusions

An analysis of five batches of the microbial pest control agent *Beauveria bassiana* strain PPRI 5339 determined the mean active substance concentration to be 1.2 x CFU/g. A screen for pathogens detected no contamination from *Staphylococcus aureus*, *Escherichia coli* or other coliforms, *Salmonella*, *Vibrio cholerae*, *Shigella* or anaerobic bacteria in any batch.

MP 4.1/02

Report: Whittaker, M (2014), Detection and enumeration of *Beauveria bassiana* and microbial contaminants in five production batches of BROADBAND microbial pest control product. APIS, Knaresborough Technology Park, Manse Lane, Knaresborough, North Yorkshire HG5 8LF. Study Number APIS-BASF-011.

Guidelines: OECD ENV/JM/MONO(2011)43.

GLP: Yes

Summary

This study was conducted to determine the concentration of the active ingredient *Beauveria bassiana* and other microbial contaminants in five production batches of the Microbial Pest Control Product (MCPC) test item Broadband. The mean enumeration result was 7.1 x CFU/g MPCP. There was no contamination by yeasts and moulds, anaerobic bacteria, *Listeria*, *Staphylococcus aureus*, *Escherichia coli* or other coliforms, *Enterobacter*, *Salmonella*, *Vibrio cholera* or *Shigella*.

Material and Methods

Test Item

Reference Name:	BROADBAND
Label Identification:	P1370
Label Identification:	P1371
Label Identification:	P1372
Label Identification:	P1373
Label Identification:	P1374
Nominal purity:	>4.0 × CFU/mL
Stability:	Stable at room temperature or under refrigeration

Germination test

1.0 mL of each bath was pipetted into 99.0 mL sterile phosphate buffered saline (PBS), sonicated for 3 minutes and allowed to stand for 30-60 minutes. 0.1 v of the diluted sample was plated onto each of three Rose Bengal agar plates. Plates were incubated for 18 hours at 28 °C.

Following incubation, a 2 cm square was cut out of the agar and placed onto a glass microscope slide. A drop of immersion oil was placed directly onto the agar and allowed to settle for 5-10 minutes before colonies were counted at 1000x magnification. Spores were considered germinated when the germ tube equalled the spore diameter. Both germinated and non-germinated spores were counted until the tally of both reached 300. Percentage viability was calculated as follows:

$$\% \text{ viability} = (G \times 100) / (G+N)$$

Where: G = germinated spores
N = non-germinated spores

Total spore count

1.0 mL of each batch was pipetted into a weighing boat and 1.0 mL of G-49 surfactant added, mixing to form a homogeneous paste. The paste was washed into a 100 mL volumetric flask and made up to the mark with deionised water. The spore suspension was sonicated for 5 minutes and then shaken well to ensure the spores were evenly dispersed. A haemocytometer was set up and filled with the spore suspension by capillary action, and then left to stand for 25-30 minutes.

The number of spores were recorded and the average of the total counts calculated. The total spore count per mL of the sample was calculated.

The method was validated by carrying out the procedures above five times sequentially on batch P1373 to demonstrate the suitability of the test method for enumerating the test item.

Contaminant screen

For all contaminants with detection limits stated in 25 mL, a stock suspension was prepared by adding at least 25 mL of each batch of MPCP to 225 mL of sterile PBS.

1. Yeasts and moulds

1.0 mL of the stock suspension was inoculated onto each of nine Yeast and Mould Agar (YMA) plates. A further six plates were inoculated with reference cultures of *Candida albicans* (three plates) and *Aspergillus brasiliensis* (three plates). Plates were incubated at approximately 26 °C for approximately 48 hours and then observed for growth.

2. *Listeria*

1.0 mL of the stock suspension was inoculated onto each of nine Listeria Selective Agar (LSA) plates. A further three plates were inoculated with a reference culture of *Listeria monocytogenes*. Plates were incubated at 37 °C for approximately 48 hours and then observed for growth.

3. *Staphylococcus aureus*

1.0 mL of the stock suspension was inoculated onto each of nine CHROMAgar Staph aureus (CASA) plates. A further three plates were inoculated with a reference culture of *Staphylococcus aureus*. Plates were incubated at approximately 37 °C for approximately 24 hours and then observed for growth.

4. *Escherichia coli* and other coliforms

A preliminary screen was conducted with a Colilert-24 test kit using a reference culture of *E. coli* as a positive control. The contents of a Colilert test vial and 1.0 g of test substance were added to 100 mL of deionised water in a sterile non-fluorescing vessel, capped, shaken and then incubated at 35 +/- 1.0°C for 24 hours, after which vessels were examined under UV light. Yellow fluorescence indicates a positive result.

The presence of *E. coli* was assessed by plating 1.0 mL of the stock suspension onto each of nine CHROMAgar E. coli (CAEC) plates. A further three plates were inoculated with a reference culture of *E. coli*. Plates were incubated at approximately 37 °C for approximately 24 hours and then observed for growth.

5. *Salmonella*

1.0 mL of the stock suspension was inoculated onto each of nine CHROMAgar Salmonella Plus (CASPA) plates. A further three plates were inoculated with a reference culture of *Salmonella bongori*. Plates were incubated at approximately 37 °C for approximately 24 hours and then observed for growth.

6. *Vibrio*

Approximately 25 mL of each batch of test item was weighed into a sterile flask containing 225 mL of alkaline peptone water. The flask was thoroughly mixed for approximately 2 minutes and then incubated at 37 °C for 7 hours. A 3 mm loop from the surface pellicle was streaked onto each of nine CHROMAgar Vibrio (CAVIB) plates. A further three plates were inoculated with a reference culture of *Vibrio alginolyticus*. Plates were incubated at approximately 37 °C for approximately 24 hours and then observed for growth.

7. *Shigella*

25 mL of test item was weighed into 225 mL of *Shigella* broth, prepared according to the manufacturer's instructions, to which 0.5 µg/ mL novobiocin had been added following autoclaving. The suspension was held for 10 +/- 2 minutes at room temperature and shaken periodically. The supernatant was poured into a sterile 500 mL Erlenmeyer flask and the pH adjusted to 7.0 +/- 0.2 with sterile 1 N NaOH.

The flasks were placed in a gas bag containing an AnaeroGen sachet to provide an anaerobic atmosphere and incubated at approximately 44 °C for approximately 18 hours, after which the enrichment culture suspension was agitated and streaked onto nine Hektoen Enteric Agar (HEA) plates. A further three plates were inoculated with a reference culture of *Shigella boydii*. Plates were incubated at approximately 37 °C for approximately 22 hours and then observed for growth.

8. *Enterobacter*

1.0 mL of the stock suspension was inoculated onto each of nine Violet Red Bile Glucose Agar (VRBGA) plates. A further three plates were inoculated with a reference culture of *Enterobacter cloacae*. Plates were incubated at approximately 37 °C for approximately 24 hours and then observed for growth.

9. Anaerobic plate count

1.0 mL of the stock suspension was inoculated onto each of nine Anaerobe Basal Agar (ABA) plates. A further three plates were inoculated with a reference culture of *Bacteroides fragilis*. Plates were placed into a gas bag containing an AnaeroGen sachet to maintain a high CO₂ environment and then incubated at approximately 37 °C for approximately 24 hours and then observed for growth.

Method Validation

The results of the validation of the method for total spore counts are shown in Table 5.1/01-1. There was no significant difference between the enumerations (ANOVA, p = 0.05, ns) and the method was deemed suitable for the detection and enumeration of the test item in the MPCP.

Table 5.1/01-1: Results of method validation for total spore count of *Beauveria bassiana* in BROADBAND MPCP

Square	Haemocytometer counts				
	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5
A1	298	316	288	291	352
A2	297	300	293	296	308
A3	312	297	233	280	302
A4	302	341	266	336	314
A5	328	298	304	313	298
B1	315	237	278	333	325
B2	299	218	297	361	308
B3	354	255	362	357	306
B4	316	312	330	344	362

B5	329	326	272	312	308
Mean	315.00	290.00	292.30	322.30	318.00
SEM	5.71	12.69	11.20	8.90	6.98
rSEM	1.81	4.38	3.83	2.76	2.20

Results

Germination test

The results of the germination tests are shown in Table 5.1/01-2. The mean result was 96.3% germination. Germination rates for each batch were included in the spore count calculation to derive an enumeration value in CFU/mL for each batch.

Table 5.1/01-2: Germination test results of *Beauveria bassiana* strain PPRI 5339 for five production batches of BROADBAND MPCP

Batch	G	N	Total	%
P1370	294	6	300	98.0
P1371	289	11	300	96.3
P1372	281	19	300	93.7
P1373	292	8	300	97.3
P1374	288	12	300	96.0
			Mean	96.3
			SEM	0.74
			rSEM	0.77%

Total spore count

The mean spore count was multiplied by the germination to derive the final enumeration value in CFU/mL.

Table 5.1/01-3: Results of total spore count of *Beauveria bassiana* strain PPRI 5339 for five production batches of BROADBAND MPCP.

Batch	Haemocytometer total count per side		Mean	Count adjusted for % germination (CFU/mL)
	A	B		
P1370	1463	1443	1453	7.1 ×
P1371	1334	1450	1392	6.7 ×
P1372	1558	1433	1495.5	7.0 ×
P1373	1468	1587	1527.5	7.4 ×
P1374	1547	1371	1459	7.0 ×
			Mean	7.1 ×
			SEM	1.2 ×
			rSEM	1.7%

Contamination screen

There was no contamination by yeasts and moulds, anaerobic bacteria, *Listeria*, *Staphylococcus aureus*, *Escherichia coli* or other coliforms, *Enterobacter*, *Salmonella*, *Vibrio cholera* or *Shigella* in any of the batches tested.

Conclusions

The validation of this method demonstrates that the method was appropriate for the analysis of *Beauveria bassiana* in the MPCP, and by extrapolation with the production batches of the MPCA. An analysis of five batches of the microbial pest control product BROADBAND determined the mean active substance concentration to be $7.1 \times \text{CFU/mL MPCP}$. A screen for microbial contaminants detected no yeasts and moulds, anaerobic bacteria, *Listeria*, *Staphylococcus aureus*, *Escherichia coli* or other coliforms, *Enterobacter*, *Salmonella*, *Vibrio cholera* or *Shigella* in any batch.

MA 4.1/03

Report: Berry, S (2012), Molecular Identify of the South African *Beauveria bassiana* isolate, strain PPRI 5339. Becker Underwood, Mahogany Ridge, KZN, South Africa and Becker Underwood, NSW 2250, Australia. Report Date: 28.11.12

Guidelines: None

GLP: Yes

Summary

Identification of *Beauveria bassiana* strain PPRI 15339 was confirmed by sequencing of the ITS ribosomal region including the 3' end of the 18S region, the ITS1 region, the 5.8S region and the ITS2 region.

The resulting sequence was BLASTED against sequences deposited in GenBank ([://www.ncbi.nlm.nih.gov/Blast](http://www.ncbi.nlm.nih.gov/Blast)). Results showed that the isolate PPRI 5339 was most related (99-100% identity) to sequences deposited as *Beauveria bassiana*.

The sequences of *Beauveria bassiana* strain PPRI 5339 was then compared with that of two competitor isolates viz. Botanigard® (*B. bassiana* isolate GHA, accession number: JN379811.1) and Naturalis® (*B. bassiana* isolate ATCC74040, accession number: FJ972972.1) The sequences of the three isolates were found to be 98-99% similar to each other and can be considered near identical species.

MA 4.1.1 Methods for the determination of relevant metabolites

Method OA02266

MA 4.1.1/01

Report: Cheng, KM (2013), Assay to determine Beauvericin within *Beauveria bassiana* concentrate with associated validation. Oxford Analytical Ltd, A3, Telford Road, Bicester, Oxfordshire OX26 4LD. Study Number OA02266.

Guidelines: SANCO/3030/99 rev 4.

GLP: Yes

Material and Methods

Approximately 1000 mg of sample was accurately weighed into a 50 mL volumetric flask, made to volume with acetonitrile and sonicated for 5 minutes. The samples was allowed to cool and then centrifuged at 3000 rpm for 5 minutes. The sample was then filtered through a Nylon 0.45 µm syringe filter. The resulting solution was analysed by LC/MS. Four ions were monitored using an MSD detector (784.5, 801.5, 806.5, 843.5 m/z). Samples were quantified against a primary standard of Beauvericin (Batch No. BCBG3253V, purity 99.0 %).

The linearity of the method was determined over the range 0.002 to 0.2 µg/mL beauvericin.

The precision of the method was determined by injecting a 0.1 µg/mL beauvericin standard into the LC/MS system six times and the sample precision was determined by injecting a spiked sample six times into the system.

One sample was spiked with 5 mL of a 1 µg/mL beauvericin standard (equivalent to ~ 5 mg/kg beauvericin in the sample) and then prepared according to the procedure above. This sample was analysed by 5 separate injections into the LC/MS system to determine the accuracy of the method.

The low level accuracy of the method was determined by spiking a sample with 0.5 mg/kg beauvericin (equivalent to ~ 0.5 mg/kg beauvericin in the sample).

Results

A summary of the validation results for the determination of beauvericin in *Beauveria bassiana* concentrate is shown in Table 4.1/03-1.

Table 4.1.1/01-1: Summary of validation results for the determination of Beauvericin in *Beauveria bassiana* concentrate

Criteria	Beauvericin
Specificity a) retention time match b) interference	RT times match No interferences noted
Confirmation of analyte identify a) molecular mass	Matches the reference standard
Linearity	R = 0.99998, n = 2 x 5
IP-Standard	%RSD = 0.45, n = 6
IP-Sample	%RSD = 2.33, n = 6
Accuracy (5 mg/kg)	%RSD = 1.63, n = 5

	Range 104 - 101 %, Mean = 98.6%
Low Accuracy (0.5 mg/kg)	%RSD = 1.94, n = 5 Range 104 – 109 %, Mean = 106%
Precision (repeatability)	< LOQ, assessed from the Accuracy
LOD	0.01 mg/kg
LOQ	0.5 mg/kg

Conclusions

This method is considered acceptable for the determination of beauvericin in *Beauveria bassiana* concentrate.

MA 4.2 Methods to Determine and Quantify Residues (Viable or Non-Viable)

Residue analytical methods for *Beauveria bassiana* PPRI 5339 are not considered necessary as no maximum residue levels have been set or requested.

Beauveria bassiana
strain PPRI 5339

DOCUMENT M-MA, Section 5

EFFECTS ON HUMAN HEALTH

Version

Date	Data points containing amendments or additions and brief description	Document identifier and version number

¹ It is suggested that applicants adopt a similar approach to showing revisions and version history as outlined in SANCO/10180/2013 Chapter 4 How to revise an Assessment Report

Table of Contents

MA 5	EFFECTS ON HUMAN HEALTH	4
MA 5.1	Basic Information	4
MA 5.1.1	Medical Data	5
MA 5.1.2	Medical surveillance on manufacturing plant personnel	5
MA 5.1.3	Sensitisation/allergenicity observations, if appropriate	5
MA 5.1.4	Direct observation, e.g. clinical cases	6
MA 5.2	Basic Studies	12
MA 5.2.1	Sensitisation.....	21
MA 5.2.2	Acute toxicity, pathogenicity and infectiveness	25
MA 5.2.2.1	Acute oral toxicity, pathogenicity and infectiveness.....	25
MA 5.2.2.2	Acute inhalation toxicity, pathogenicity and infectiveness.....	31
MA 5.2.2.3	Intraperitoneal/subcutaneous single dose.....	43
MA 5.2.3	Genotoxicity Testing.....	46
MA 5.2.3.1	<i>In Vitro</i> studies	46
MA 5.2.4	Cell Culture Study	52
MA 5.2.5	Information on Short-Term Toxicity and Pathogenicity.....	52
MA 5.2.5.1	Health effects after repeated inhalatory exposure.....	52
MA 5.2.6	Proposed treatment: first aid measures, medical treatment.....	52
MA 5.3	Specific Toxicity, Pathogenicity and Infectiveness Studies	52
MA 5.4	<i>In Vivo</i> Studies in Somatic Cells	53
MA 5.5	Genotoxicity – <i>In Vivo</i> Studies in Germ Cells	53
MA 5.6	Summary of Mammalian Toxicity, Pathogenicity and Infectiveness and Overall Evaluation	54

MA 5 EFFECTS ON HUMAN HEALTH

Several key documents were used in the preparation of this submission: Draft Assessment for *Beauveria bassiana* strain ATCC , Draft Assessment for *Beauveria bassiana* strain , Zimmermann (2007) (Doc K-MA 5/01) and EFSA Scientific Opinion on the risks to human and animal health related to the presence of beauvericin and enniatins in food and feed (2014)⁴.

MA 5.1 Basic Information

PPRI 5339 is a naturally occurring, non-modified strain of *B. bassiana* strain, originally isolated by Dr Schalk Schoeman from the larva of a tortoise beetle, *Conchyloctenia punctata* (Coleoptera: Cassidinae) collected in Escombe, Queensburgh, KwaZulu Natal, South Africa. Further information on origin can be found in MA, Point 2.1.2. *B. bassiana* was identified as a rare pathogen in humans but in these few and very specific cases the strain was not characterised. Furthermore, allergenicity in humans has been reported. This fungal species is not closely related to any known microorganism that has to be regarded as a regular human or mammalian pathogen. A further, more general argument against infectivity and pathogenicity to man is that most *B. bassiana* strains do not survive and replicate at temperatures higher than but this, of course, does not exclude the possibility of at least local effects that may be caused by a microorganism just after it has entered the host body.

At the time of writing, two DARs have been produced for *B. bassiana*, strains ATCC 74040¹ and GHA². Strain PPRI 5339 was found to be 98-99% similar to the above, following ribosomal sequencing, see Point MA 1.3.1 and therefore strain PPRI 5339 can be considered essentially identical to strains ATCC74040 and GHA. As such the major conclusions reported in the DARs for strains ATCC74040 and GHA are equally applicable to strain PPRI 5339 and are reproduced where necessary in this submission. The body of information in the open literature regarding the toxicity of *B. bassiana* is extensive. The summation of these data are provided in detail in the review by Zimmermann, 2007³ (Doc K-MA 5/01) and form the basis of the following discussions, supplemented with any other relevant literature that has subsequently been published.

Additionally, relevant toxicity data on the main metabolite of *B. bassiana* beauvericin is summarised in detail in the EFSA Scientific Opinion on the risks to human and animal health related to the presence of beauvericin and enniatins in food and feed (2014)⁴. The data and major conclusions reported in this Scientific Opinion document are applicable to this submission

¹ Draft Assessment Report (DAR) (2008) Initial Risk Assessment Provided by the Rapporteur Member State Germany for the Existing Active Substance Beauveria Bassiana ATCC 74040 of the Fourth Stage of the Review Programme Referred to in Article 8(2) of Council Directive 91/414/EEC. Volume 3, Annex B, part 2, B.6. August 2008.

² Draft Assessment Report (DAR) (2008) Initial Risk Assessment Provided by the Rapporteur Member State Germany for the Existing Active Substance Beauveria Bassiana GHA of the Fourth Stage of the Review Programme Referred to in Article 8(2) of Council Directive 91/414/EEC. Volume 3, Annex B, part 2, B.6. August 2008.

³ Zimmermann, G (2007) Review on safety of the entomopathogenic fungi Beauveria bassiana and Beauveria brongniartii. Biocontrol Science and Technology, (2007) 17(5-6): 553-596.

⁴ EFSA Scientific Opinion on the Risks to Human and Animal Health Related to the Presence of Beauvericin and Enniatins in Food and Feed. EFSA Journal 2014; 12(8):3802.

and will be reproduced where necessary.

Specific studies for infectivity, pathogenicity and toxicity have been performed with strain PPRI 5339 in laboratory animals and are reported under Sections M-MA 5.2 to 5.6, supplemented with other relevant data where applicable. These studies clearly proved that this strain was not infective and not pathogenic, at least following single exposure, irrespective of the administration route. Some toxicity was observed following inhalation and intratracheal application. There are also indications for allergy by inhalation and by skin contact in humans. In this section, available experience with *B. bassiana* in humans is also summarised and evaluated.

MA 5.1.1 Medical Data

No epidemiological studies on the presence of *B. bassiana* in humans or on a possible relationship with diseases in humans have been performed. Thus, experience in humans is based on medical surveillance during manufacturing, a few anecdotal reports from the open literature on the isolation of *B. bassiana* from patients suffering from different diseases and a small number of studies on the occurrence of this micro-organism in neonates and on allergenicity in humans.

MA 5.1.2 Medical surveillance on manufacturing plant personnel

No evidence of adverse health effects have been observed in any of the employees involved in the production, handling and application of *B. bassiana* strain PPRI 5339. However, microbial products are generally expected to be skin sensitisers, due to the high protein content. It is therefore important that employees, workers and operators handling *B. bassiana* strain PPRI 5339 wear appropriate protective equipment.

MA 5.1/01

Report: Neethling, K (2013) Memorandum: Exposure to, and adverse health effects from, exposure to *Beauveria bassiana* strain PPRI 5339 during manufacturing. BASF Agricultural Specialities (PTY) Ltd., 91 Clifton Caanyon Drive, Clifton Farm Stockville Quarry, Mahogany Ridge, KwaZulu Natal, South Africa. November 2013.

Summary

BASF Agricultural Specialities (PTY) Ltd (formerly Becker Underwood South Africa (Pty) Ltd. and before that Biological Control Products (Pty) Ltd.) manufactures *B. bassiana* strain PPRI 5339. The company started producing this strain in October 2002 and to date has made 1.01 tons of technical grade material (23,580 liters of formulated product).

During this time, no evidence of any adverse health effects have been observed by those individuals producing, processing, packaging, analyzing, handling, applying or otherwise exposed to *B. bassiana* strain PPRI 5339 technical grade material or formulated products.

MA 5.1.3 Sensitisation/allergenicity observations, if appropriate

Westwood et al. (2005) (Doc K-MA 5.1.3/01) used crude extracts of *B. bassiana* strain ATCC 90517 for immunoblotting with pooled as well as individual human sera and found IgE binding

of a number of potential allergens present in the extracts. The authors reported evidence of *B. bassiana*-specific proteins that might cause allergies as well as indication for cross-sensitisation because of certain reactive epitopes that this microorganism has in common with other fungi such as *Alternaria alternata* or *Aspergillus fumigatus*. In the *in vivo* part of this study, ten people were intradermally injected 0.1 mL of a dialysed (against 0.15 N NaCl) and filtered crude *B. bassiana* extract and monitored for 15 - 30 min for the development of allergenic reactions. Control persons received saline and histamine. Seven out of the 10 participants treated with the extract displayed skin reactions. Although this may be not considered entirely convincing since it is sometimes difficult to distinguish between skin reaction because of sensitisation and irritation, it must be seriously taken into account that four of five people who had reported previously occupational exposure to *B. bassiana* showed a positive skin reaction as well as bands suggesting reactivity in the Western blot.

In a study in the Netherlands, Beaumont *et al.* (1985a & 1985b) (Doc K-MA 5.1.3/02 and Doc K-MA 5.1.3/03) examined patients with recurrent allergic bronchial obstruction and identified *B. bassiana* (strain not specified) as an allergen by means of intracutaneous challenge with strong reactions in 5 of 73 test persons (6.8 %). This percentage was higher than seen with most other fungal species included in this investigation although the presence of *B. bassiana* spores in air samples was low.

Henke *et al.* (2002) (Doc K-MA 5.1.3/014) reported allergic alveolitis in an immunocompromised patient suffering from disseminated infection with *Beauveria spp.* (see below).

Semalulu *et al.* (1992) (Doc K-MA 5.1.3/05) cited references (not available in the DAR for *B. bassiana* strains GHA and ATCC) that claimed occurrence of moderate or even severe allergenic reactions among scientists working with *B. bassiana* but also mentioned papers suggesting that there were no deleterious effects in workers handling this fungus.

Taking into consideration the presented information from the open ^{2,3} and observations in laboratory animals presented herein, there is enough evidence to consider *B. bassiana*, irrespective of the strain, a potential human allergen by both skin and inhalatory contact.

MA 5.1.4 Direct observation, e.g. clinical cases

There is evidence from the open ^{2,3} that *B. bassiana* (strain never specified) was isolated in few cases from people who suffered from various diseases.

Eye infections

Low *et al.* (1997) (Doc K-MA 5.1.4/01) described the case of a healthy 67 years old woman in Australia who experienced *B. bassiana*-related keratitis following mechanical injury of the cornea. Pain in the affected eye, redness, epiphora, and decreased vision were the initial symptoms. Ten days later, a deep peripheral corneal infiltrate containing a faint linear body resembling a caterpillar hair had developed. After aspiration of a filamentous bundle from the posterior surface of the cornea, smears were prepared and media for bacterial and fungal growth inoculated. Hyphae were seen in Gram and Giemsa stains. While treatment with chloramphenicol drops had no effect, signs improved when prednisolone phosphate and homatropine were topically applied. After recognition of a fungal infection, the patient received miconazole and natamycin but infiltration and intraocular pressure increased markedly, posterior

synechiae formed, and vision decreased to hand movements. Eventually, surgical dissection of the peripheral cornea and complete removal of infected tissue as a deep block (approximately 3 x 2 mm of tissue) was successful. The patient improved rapidly and was discharged from hospital. Five months after hospitalisation, the eye was quiet with one small area of posterior synechiae. The pressure was normal, and the vision had become much better. Sections of the removed corneal tissue were plated onto Sabouraud's agar and into Sabouraud's broth. After 4 days' incubation, powdery white colonies appeared on the Sabouraud's agar from both the anterior chamber and corneal specimens and puff-balls became visible in the Sabouraud's broth. Small clusters of conidiogenous cells were seen under a light microscope, whose ends tapered in a zigzag fashion, a spore appearing at each bend. These features were considered indicative of *B. bassiana*.

Kisla *et al.* (2000) (Doc K-MA 5.1.4/02) observed an ocular infection in an 82-year-old woman with a corneal graft following optical surgery because of blunt trauma to her right eye. The patient was examined and sutures were removed about half a year after the intervention. One month later, she complained of decreased vision, a foreign body sensation, and mild aching in the affected eye. A sectoral area of oedema within the graft was observed. The patient was treated with topical prednisolone acetate. Six days later, she complained of further decrease in vision, photophobia, tearing, and pain. Vision had impaired to perception of hand movements. The corneal graft was diffusely oedematous with an inferior epithelial defect but with no infiltrate. The prednisolone acetate treatment was discontinued and gentamicin ointment was administered every two hours but the patient's symptoms persisted. The right eye became profoundly injected with increased corneal oedemas and a substantial anterior chamber inflammatory response. A stromal infiltrate was noted in the area of the epithelial defect. Smears stained with Giemsa revealed the presence of polymorphonuclear leukocytes and septate hyphae. Appropriate media were inoculated for cultures of bacteria and fungi. Bacterial cultures yielded no growth while on the fungal media 14 colonies of a white mould grew that were provisionally identified as a *Beauveria* species. In another laboratory, the fungus was later identified as *B. bassiana* by its colony morphology and microscopic features. Therefore, therapy with gentamicin was discontinued and the patient was given a topical treatment with natamycin and oral fluconazole. Some progress was seen but vision remained severely compromised. Approximately four months after initial symptoms, the patient underwent a repeat penetrating keratoplasty. Histopathological examination of the excised tissue revealed the presence of corneal stromal thinning, oedema, vascularisation, and scarring. Gomori's methenamine silver and Brown & Hopps stains were negative for fungal elements. Eleven months later, the patient's postoperative course was uneventful. The corneal graft was clear and vision much better although macular degeneration changes were present.

Sachs *et al.* (1985) (Doc K-MA 5.1.4/03) reported infection and eventually penetration of the cornea by *B. bassiana* in a 64-year old farmer from Massachusetts. Infection occurred after surgical removal of a foreign body from the cornea and might have been potentiated by parallel use of antibiotics such as gentamicin and steroids (hydrocortisone, dexamethasone) after surgery.

A case of corneal ulceration in the cause of an eye infection with *B. bassiana* was described by Ishibashi *et al.* (1984) (Doc K-MA 5.1.4/04).

MA 5.1.4/05

-
- Report:** Figueira, L., Pinheiro, D., Moreira, R., Pinto, E., Simões, J., Camisa, E., Torrão, L., Palmares, J., Falcão-Reis, F. (2012) *Beauveria bassiana* keratitis in bullous keratopathy: antifungal sensitivity testing and management. *Eur J Ophthalmol* 2012; 22 (5): 814-818.
- Guidelines:** Not applicable
- GLP:** Not applicable

Summary

The authors reported the first case of *B. bassiana* keratitis in a patient with aphakic bullous keratopathy. The fungal keratitis proved to be highly resistant to topical clotrimazole. Molecular identification was based on DNA sequence analysis. The minimal inhibitory concentrations (MIC) obtained were 2 µg/mL for voriconazole, 0.250 µg/mL for posaconazole, and >128 µg/mL for fluconazole; amphotericin B MIC was >16 µg/mL. In the absence of clinical improvement, a penetrating keratoplasty (PK) was performed. The patient was discharged on topical and systemic voriconazole and prednisolone 40 mg PO/day. The eye remained calm with a transparent cornea and clear anterior chamber.

B. bassiana keratitis is extremely rare, with only a few cases reported. Its risk factors are still unclear. This study reported the first case in a patient with aphakic bullous keratopathy, which proved highly resistant to antifungal therapy (antifungal susceptibility results are presented). A PK was necessary for clinical improvement. A further review of the literature was performed in an effort to define therapeutic strategies.

MA 5.1.4/06

- Report:** Sonoyama H, raki-Sasaki K, Kazama S, Kawasaki T, Ideta H, Sunada A, Asari S, Inoue Y, Hayashi K (2008) The characteristics of keratomycosis by *Beauveria bassiana* and its successful treatment with antimycotic agents. *Clin. Ophthalmol.*, 2(3): 675-678.
- Guidelines:** Not applicable
- GLP:** Not applicable

Summary

An 80-year-old woman struck her left eye with the frame of her glasses. Due to continuing ocular pain and hyperemia, she was referred to our hospital 9 days after the original injury. The patient was also suffering from recurrent diabetic iritis and continuously used topical antibiotics and corticosteroids. At the time of her first visit to our hospital, there was ulceration of the corneal epithelium at the 5 o'clock region along with slight superficial infiltration, slight oedema, and ciliary injection. No endothelial plaques or any immune rings were noted. Although a part of the Descemet's membrane was folded, there was only slight anterior chamber inflammation. A corneal scraping smear indicated the presence of Gram-positive fungal septate hyphae with budding, which led to a diagnosis of keratomycosis by filamentous fungus. Positive staining by Fungiflora Y also supported the keratomycosis diagnosis. From the culture of the corneal scraping, we were able to isolate a whitish yellow colony, with the fungus exhibiting zigzag rachis and oval conidia, which are characteristics of *B. bassiana* in slide cultures. Two

different microbiology laboratories, the Department of the Laboratory for Clinical Investigation at Osaka University Hospital and the Chiba University Research Center for Pathogenic Fungi and Microbial Toxicoses, independently confirmed the presence of the organism. Any other organisms were detected by the culture of corneal scraping.

MA 5.1.4/07

Report: Tu EY and Park AJ (2007) Recalcitrant *Beauveria bassiana* keratitis: confocal microscopy findings and treatment with posaconazole (Noxafil). *Cornea*, 26(8): 1008-1010.

Guidelines: Not applicable

GLP: Not applicable

Summary

A 58-year-old woman was referred to the University of Illinois Eye and Ear Infirmary (IEEI) for evaluation of a recalcitrant central corneal ulcer in the right eye for the past month. She was an Acuvue II soft contact lens (Johnson & Johnson, Jacksonville, FL) wearer and reported good lens hygiene habits with Opti-Free solutions (Alcon, Fort Worth, TX). There was no history of trauma, but the patient kept a rose garden and lived near several golf courses. Before presentation, various combinations of Vigamox, Tobradex, prednisolone acetate, and subsequently, trifluridine and scopolamine, were tried without improvement. On presentation to the Wheaton Eye Clinic, corneal cultures were obtained, and fortified topical vancomycin 50 mg/mL and tobramycin 14 mg/mL were initiated hourly for 3 days without improvement. Cultures and smears returned negative, and she was referred to the IEEI for further evaluation. The patient was extremely uncomfortable, with severe photophobia and a best-corrected vision of 20/400 and 20/40 in the right and left eyes, respectively. The left eye appeared normal. The right eye exhibited a shallow 3 x 4 mm central ulcer, minimally necrotic, with a clear corneal periphery and no hypopyon. Confocal microscopy (Confoscan 3, Nidek, Japan) and corneal scrapings for Diff-Quick smear (DIFCO, Detroit, MI) and culture were obtained.

Confocal microscopy revealed extensive filamentary forms consistent with a filamentous keratitis. A corneal scraping was performed for culture and Diff-Quick stain, which confirmed the presence of a filamentous organism. Natamycin 5% was added hourly with a discontinuation of antibacterial medications. Fluconazole 200 mg daily was added 3 days later with no change in clinical appearance or symptoms over the next 8 days. Because fungal cultures remained negative, topical Bactrim was added for the next week to cover possible *Nocardia* keratitis. Topical voriconazole 1% was added when fungal cultures revealed a *Beauveria* species after progressive worsening on a regimen of topical natamycin/oral fluconazole for 18 days. For the next 4 days, the patient had worsening pain on the 3-drug regimen, necessitating the addition of oral posaconazole 200 mg 4 times daily and discontinuation of the fluconazole. The *Beauveria* sp. was later identified as *B. bassiana* resistant to amphotericin B.

In summary, these ophthalmological case reports do not point to a special risk of *B. bassiana* strain ATCC to cause eye infections in operators since adverse effects were only seen after damage to the cornea that allowed invasion of the fungus into the eye. There are some reports on keratitis or other ocular disease in humans with intact cornea, but these events are rare. The findings suggest a possible affinity of *B. bassiana* to ocular tissues when the cornea once had

been penetrated. This was partly confirmed by Ishibashi *et al.* (1987) (Doc K-MA 5.1.4/04) who reported local corneal infection after experimental ocular infection of rabbit eyes. (minimum inhibitory concentration [MIC], 8 mg/mL) but most likely susceptible to capsosungin (MIC, 0.125 mg/mL), voriconazole (MIC, 0.25 mg/mL), and posaconazole (MIC, 0.25 mg/mL). Progressive improvement was seen in clinical appearance and pain over the subsequent 19 days, at which point the natamycin was discontinued. During the course of treatment, the patient developed pleural effusions and elevated Liver Function Tests (LFTs) thought to be possibly related to posaconazole, which necessitated discontinuation of the drug after 1 month of treatment. At this point, the keratitis was judged to have completely healed. The LFTs and pleural effusions resolved after the drug was discontinued.

It was unclear what the role contact lens wear had in our case. This has not been previously reported, and the patient's contact lens paraphernalia was not available for fungal testing.

Systemic disease

Henke *et al.* (2002) (Doc K-MA 5.1.4/08) reported disseminated infection with *Beauveria spp.* in a 38-year-old woman who had been diagnosed to have extramedullary acute myeloid leukaemia and was successfully treated by chemotherapy. Two weeks after discharge from hospital, the patient was readmitted because of severe dyspnea, dry cough, pain in the right upper abdomen, and fever. Lung function was impaired and a CT scan of the thorax revealed a discrete interstitial infiltrate. Allergic alveolitis was histologically confirmed by transbronchial lung biopsy. Ultrasonography of the abdomen revealed multiple lesions in the liver and spleen, suggesting systemic fungal infection. In liver biopsy samples, extensive focal necrosis was recorded. Samples from these specimens, verified cultures of *Beauveria bassiana* and of the related species *Beauveria brongniartii* were cultured, compared, and gave similar results, but genotyping (sequencing) pointed rather to the latter one. The woman patient was treated with steroids and, following microbiological and pathological findings of fungal infection, antifungal therapy was initiated with itraconazole. This was successful and, three weeks later, the patient had recovered.

A systemic infection with *Beauveria bassiana* in a 44-year-old woman from a rural area who underwent chemotherapy for treatment of acute lymphoblastic leukemia was reported by Tucker *et al.* (2004) (Doc K-MA 5.1.4/09). First signs of infection (fever, neutropenia) appeared on day 15 of cytostatic treatment. Few days later, small (< 1 cm) purple macula "cigarette burn"- like lesions were noted on the left upper arm. Skin lesions progressed, involving the patient's arms, legs, buttocks, and face, and became necrotic and exudative. Histopathological examination of skin biopsy specimen revealed sharply demarcated areas of necrosis with lack of cellular reaction at the interface. The necrotic tissue was heavily permeated by fungal hyphae, which also invaded the local blood vessels. The isolate was identified as *Beauveria bassiana* due to morphological, physiological, and growth characteristics. This finding was confirmed by gene sequencing.

In addition, the patient complained of symptoms of sinusitis, headache, and facial pain and had percussion tenderness over her maxillary sinuses. Later on, she developed a persistent haemorrhagic left-sided pleural effusion and there was evidence of lung necrosis. Serum transaminases were elevated from day 21 but abdominal ultrasound scan was normal.

She received first fluconazole and then intravenous amphotericin in combination with itraconazole for a further 25 days. Antifungal therapy was continued for the duration of her

neutropenia. According to the report, treatment was successful and the skin lesions continued to heal over several months with some scarring.

Gürcan *et al.* (2006) (Doc K-MA 5.1.4/10) reported isolation of *Beauveria bassiana* from a 51-year-old man who suffered from lung adenocarcinoma with penetration of the thoracic wall. In the third week after operation, empyema because of increased and turbid pleural fluid occurred. A pleural fluid sample contained 1600 leucocytes/ μL (50 % of them were polymorphonuclear cells). Additionally, blastospores and hyphae were seen in Gram and Giemsa stained smears. Fluid was cultured and *Beauveria bassiana* identified. A second operation was performed and empyema regressed within one week. Antifungal therapy was not applied. Thus, in this case, it seems not entirely clear whether the symptoms and pathological findings were actually due to the infection.

The available published information suggests that disseminated infections have been observed only in people under immunosuppression following exposure to chemotherapeutic drugs or in individuals who were severely ill. It is not likely that such persons will get into close contact with *Beauveria bassiana* when used for plant protection purposes. Health risks for operators, bystanders or workers can be rather expected to arise from the sensitising properties of this micro-organism. In particular, inhalation allergy might be a problem.

Further information on occurrence in humans and medical use of *B. bassiana*:

Lackner *et al.* (2004) (Doc K-MA 5.1.4/11) assessed the time period after birth after which fungal spores of different species could be detected in human nasal mucus. Therefore, nasal mucus samples were taken from 30 neonates immediately after birth, on the first and fourth day *post partum*, and (so far available) after two and four months. The samples were obtained with sterile cotton swabs and cultured on Sabouraud glucose agar plates at 25 °C. Fungal cultures were identified either by conventional microscopy or by molecular techniques. In order to show whether fungi in nasal mucus of newborns were acquired by contamination during birth, mucus of the maternal vagina was examined as well.

All newborns and their mothers did not show or report clinical signs of fungal disease. Just after birth, in 6 of 30 neonates fungal cultures were detected in nasal mucus. In three of them, *C. albicans* was found, probably due to contamination when passing the maternal vagina as cultures of vaginal mucus of their mothers were positive for *C. albicans* too. Another three neonates showed *Penicillium* sp., and one of these also *B. bassiana*. Positive fungal cultures were obtained in 2 of 29 or in 4 of 26 neonates on the second or fifth day of life, respectively. In all instances, fungal presence in nasal mucus was limited to one day only. After the second month of life, examination of nasal mucus yielded positive fungal cultures in 8 of 11, after four months in 17 of 18 babies with a wide array of different species. The authors considered the finding, *i.e.*, fungal positive cultures from almost all nasal mucus, after four months of life as similar to the situation in adults, and hence fungal spores should be considered a normal content of nasal mucus, which alone would not be a pathological finding. These results support the assumption of ubiquitous occurrence of *Beauveria bassiana*.

MA 5.2 Basic Studies

A summary of the basic studies submitted by the registrant is presented in the following table. The data submitted by the registrant indicates that *B. bassiana* is potentially irritating to the eye, but not irritating to the skin of laboratory animals. No evidence of acute toxicity, pathogenicity or infectiveness was observed in any of the studies.

Table MA 5.2-1: Summary of basic studies submitted for *Beauveria bassiana* strain PPRI 5539

M-MA Point	Study/Route/Method	Species	Dose per Animal	Results	Conclusion	Reference
5.2/01	Acute Eye Irritation OECD 405	Rabbit	100 mg/eye	Positive	Irritating to eye	█ (2011a)
5.2/02	Acute Eye Irritation OECD 405	Rabbit	100 mg/eye	Negative	Not irritating to eye	█ (2007a)
5.2/03	Acute Dermal Irritation OECD 404	Rabbit	500 mg per animal – semi-occlusive	Negative	Not irritating	█ (2011b)
5.2/04	Acute Dermal Toxicity OECD 402	Rat	5050 mg/kg – semi-occlusive	No evidence of toxicity.	= >5050 mg/kg	█ (2011c)
5.2/05	Acute Dermal Toxicity OECD 402	Rat	2000 mg/kg – semi-occlusive	No mortality observed. Erythema and mild oedema noted day 1, which recovered between days 2 and 4. Epithelisation was registered between days 2 and 8.	= >2000 mg/kg	█ (2007a)

In a study summarised in the DAR for *B. bassiana* strain indicated that during a dermal acute toxicity study, signs of slight but persistent local irritation was observed. In this study no mortality or signs of systemic toxicity were observed and the was > 1.6 x CFU. A further study summarised in the DAR for *B. bassiana* strain ATCC no mortality or signs of systemic toxicity were observed and the was >2000 mg/kg, although slight erythema and oedema were noted and it was concluded that the test substance was slightly irritating to the skin.

B. bassiana can be described as potentially irritating to the eyes and skin, assumed to be relevant to the species and not strain specific, but no assessment of classification is necessary as Regulation (EC) No 1272/2008 does not apply to microorganisms.

MA 5.2/01

- Report:** [REDACTED], JO, (2011a), Acute eye irritation study in rabbits. [REDACTED]
Unpublished Report No. 15370-11
- Guidelines:** US EPA OCSP 870.2400
FIFRA 7 USC 136
OECD Guidelines for the Testing of Chemicals No.: 405.
- GLP:** Yes
- Deviations:** The provided Certificate of Analysis was not accompanied by a GLP compliance statement.
Stability information was not provided to the testing facility.
Relative humidity was at times outside the protocol range. The deviations did not affect the outcome of the study.

Aim of the Study

The aim of the study was to assess the relative level of eye irritation following a single exposure of the test substance to rabbits.

Materials and methods

Day of Treatment: 22 August 2011

End of Experiment: 29 August 2011

Test Item

Name: *Beauveria bassiana* strain PPRI 5339
Batch No.: S627
Physical State: Powder
Colour: Light cream powder
Purity: 2.06 x spores/g provided information
Storage conditions: Store away from light in a cool (5 °C), dry dark place

Albino New Zealand White rabbits (provided by Rich Glo, Inc., Wills Point, TX) in acceptable health condition were used for the study. Both eyes of each animal were examined 24 hours prior to the start of the study. Only those animals without eye defects or irritation were selected for testing.

A single dose of 100mg of the test item was placed into the conjunctival sac of the right eye of each animal. The lids were held closed gently for one second to prevent the loss of the test item. The untreated left eye served as a control.

The eyes were examined at 1, 24, 48 and 72 hours then 4 and 7 days after treatment and the grades of ocular reaction recorded. The corneas of all treated eyes were examined immediately

after the 24 hour observation with a fluorescein sodium ophthalmic solution. Any of the corneas that exhibited fluorescein staining at the 24 hour observation were examined in this manner at each consecutive observation until fluorescein staining of the cornea no longer occurred. All treated eyes were washed with water for one minute immediately after recording the 24 hour observation.

The irritation of the cornea was recorded using a four point grading scale Draize *et al.* (1944) and a seven point scale was used to rate the test substance based on eye irritation. Any corneal involvement or iridic irritation with a score of 1 or more is considered positive. Any conjunctival irritation (redness or chemosis) with a score of 2 or more is considered positive.

Findings

A summary of the irritation scores is presented in the following table.

Table MA 5.2/01-1: Average (n=3) values assigned to ocular reactions after treatment of rabbits with *Beauveria bassiana* strain PPRI 5339

I. Cornea	Hours after treatment				Days after treatment	
	1	24	48	72	4	7
A. Opacity	0.0	1.0	1.0	1.0	0.0	0.0
B. Area	0.0	2.0	2.0	2.0	1.7	0.0
C. Fluorescein Staining	-	PA/PB	PA/PB	PA/PB	0.0	-
D. Stippling	0.0	0.0	0.0	0.0	0.0	0.0
II. Iris						
A. Grade	0.0	0.0	0.0	0.0	0.0	0.0
III. Conjunctiva						
A. Redness	1t	2.0	2.3	2.3	0.7	0.0
B. Chemosis	0.7	2.3	2.3	2.3	0.7	0.0
C. Discharge	1.0	2.0	2.0	2.0	0.0	0.0
D. Necrosis/Ulceration	0.0	0.0	0.0	0.0	0.0	0.0

P – Positive fluorescein staining (A – One quarter or less; B – Greater than one quarter but less than half)

Conclusions

Due to the reversible effects observed related to conjunctival redness and conjunctival oedema (chemosis), the average score for these values at 24, 48 and 72 hours post exposure is ≥ 2 , the study indicates that the test item is irritating to the eye. No classification is necessary based on this study as Regulation (EC) No 1272/2008 does not apply to microorganisms.

MA 5.2/02

Report: [REDACTED] (2007a), Acute eye irritation study of test item Bb Spore Concentrate in rabbits. [REDACTED]

Unpublished Report No. 06/338-005N

Guidelines:	OECD Guidelines for Testing of Chemicals No.: 405 EU Method B.5 US EPA OPPTS 870.2400
GLP:	Yes
Deviations	None

Aim of the Study

The objective of the study was to determine the acute eye irritation effect of the test item Bb (*B. bassiana* strain PPRI 5339) spore concentrate in New Zealand White Rabbits. The test item was applied in a single dose to the left eye of each animal. The duration of the study was sufficient to evaluate the reversibility of the effect observed.

Materials and methods

Day of Treatment: 18 January 2007

End of Experiment: 25 January 2007

Test Item

Name:	Bb spore concentrate
Batch No.:	F1096
Physical State:	Powder
Colour:	Uniform cream powder
Expiry Date:	June 2007
Storage conditions:	Store away from light in a cool (5 °C), dry dark place

New Zealand White rabbits (provided by Ferenc Sándor breeder 173 Kartal, Császár út 135, HUNGARY) in acceptable health condition were used for the study. Both eyes of each animal provisionally selected for testing were examined 24 hours prior to the start of the study. Animals showing eye irritation, ocular defects or pre-existing corneal injury were not used.

A single dose of 0.1g of the test item Bb (*B. bassiana* strain PPRI 5339) spore concentrate was administered to 3 male rabbits by instillation into the conjunctival sac of the left eye. The eyelids were held closed gently for several seconds to prevent the loss of the test item. The contralateral eye served as a control. Immediately after administration of the test item an assessment of the initial pain reaction was made according to a six point scale by Draize (1977) and OECD 405 (24 April, 2002). The eyes of the test animals were not washed out after the application of the test item.

The eyes were examined at 1, 24, 48 and 72 hours then one week after te treatment. The duration of the observation period was sufficient for the statement of reversibility or irreversibility of changes. Any clinical signs of toxicity or signs of ill-health during the study were recorded.

At the end of the observation period, all animals were sacrificed by intramuscular injections of Ketamin and Xylazin followed by i.v. Euthanyl anaesthesia.

Findings

One hour after the single application of test item Bb spore concentrate into the eyes of the rabbits slight to moderate redness, slight chemosis and severely increased discharge were observed in the eye of the test animals.

There were no effects observed in the cornea and iris during the study period.

48 hours after the treatment in one animal only slight redness was seen. The other two animals had moderate redness, slight chemosis and slightly increased discharge.

72 hours after the treatment slight to moderate redness occurred. One week after the treatment every animal was symptom-free.

At one week after treatment the study was terminated, with all animals free of symptoms of irritation.

During the study the control eyes of animals were symptom-free. The general state and behaviour of the animals were normal throughout the study period. There were no notable body weight changes during the contact and observation period.

The animals' individual mean scores (considering readings at 24, 48 and 72 hours after the treatment) are listed in Table 5.2/02-01.

Table MA 5.2/02-1: Mean scores of symptoms after treatment of rabbits with Bb spore concentrate

Symptom	Individual mean scores		
	24 hours after treatment	48 hours after treatment	72 hours after treatment
Cornea opacity	0.00	0.00	0.00
Iris	0.00	0.00	0.00
Redness	1.33	1.66	2.00
Chemosis	0.33	0.66	1.33
Discharge	0.33	1.33	1.33

Conclusion

In conclusion, test item Bb spore concentrate applied to the rabbits' eye mucosa, caused slight to severe conjunctival irritant effects, fully reversible within 1 week. No classification is necessary based on this study as Regulation (EC) No 1272/2008 does not apply to microorganisms.

MA 5.2/03

Report:

(2011b), Acute dermal irritation study in rabbits.

Unpublished Report No. 15371-11

Guidelines:

US EPA OCSP 870.2500, FIFRA 7 U.S.C. 136

OECD Guidelines for the Testing of Chemicals No.: 404

GLP: Yes

Deviations: The provided Certificate of Analysis was not accompanied by a GLP compliance statement.

Stability information was not provided to the testing facility.

Relative humidity was at times outside the protocol range. The deviations did not affect the outcome of the study.

Aim of the Study

The aim of the study was to assess the relative primary skin irritation level of the test substance *B. bassiana* strain PPRI 5339 on rabbits.

Materials and methods

Day of Treatment: 23 August 2011

End of Experiment: 26 August 2011

Test Item

Name: *Beauveria bassiana* strain PPRI 5339

Batch No.: S627

Physical State: Light cream powder

Purity: 2.06 x spores/g provided information

Storage conditions: Room temperature

Safety precautions: Routine safety and hygienic procedures will be sufficient to assure personnel health and safety

Albino New Zealand White rabbits (provided by Rich Glo, Inc., Wills Point, TX) in acceptable health condition were used for the study.

Three animals (2 male, 1 female) were selected for the study. A test site of at least 8cm x 8cm was prepared on the dorsal area of the trunk the day prior to treatment. Each test site was treated with 500 mg test item moistened with 0.5 ml DI water and covered with a semi-permeable dressing. The test substance was maintained in contact with the skin for 24 hours.

Observations for erythema and edema formation and any other dermal defects or irritation were made a 1, 24, 48 and 72 hours after removal of the dressings. Dermal irritation was scored on a four point scale (Draize technique).

Findings

The scores for this study are presented in the following table.

Table MA 5.2/03-1: Results of acute dermal irritation study in rabbits for *Beauveria bassiana* strain PPRI 5339.

Animal	Erythema	Oedema	Primary
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Number	Hours				Hours				irritation scores
	1	24	48	72	1	24	48	72	
0502-M	0	0	0	0	0	0	0	0	0.00
0504-M	1	1	0	0	0	0	0	0	0.50
0.545-F	1	0	0	0	0	0	0	0	0.25
Primary irritation index					0.3				

M - male

F - female

only the first four observations are used for calculations
based on the mean 72 hour score for erythema and edema

Conclusions

The primary irritation index of 0.3 out of a possible 8 was obtained from the observations at 1, 24, 48 and 72 hours and was used to give *B. bassiana* strain PPRI 5339 a descriptive rating of slightly irritating. No assessment of classification is necessary based on this study as Regulation (EC) No 1272/2008 does not apply to microorganisms.

MA 5.2/04

Report: [REDACTED] (2011c), Acute dermal toxicity study in rats. [REDACTED]

Unpublished Report No. 15368-11

Guidelines: US EPA OCSP 870.1200
FIFRA 7 USC 136
OECD Guidelines for the Testing of Chemicals No.: 402

GLP: Yes

Deviations: The provided Certificate of Analysis was not accompanied by a GLP compliance statement.

Stability information was not provided to the testing facility.

Relative humidity was at times outside the protocol range. The deviations did not affect the outcome of the study.

Aim of the Study

The aim of the study was to assess the systemic toxicity potential and relative skin irritancy of the test substance when administered to rats.

Materials and methods

Day of Treatment: 25 August 2011

End of Experiment: 8 September 2011

Test Item

Name: *Beauveria bassiana* strain PPRI 5339

Batch No.: S627

Physical State:	Light cream powder
Purity:	2.06 x spores/g provided information
Storage conditions:	Room temperature

Albino Sprague-Dawley rats (provided by Texas Animal Specialities, Humble, TX) in acceptable health condition were used for the study.

Ten animals (5 male, 5 female) were selected for the study. A test site of at least 10% of the total body surface area was prepared on the dorsal surface of the trunk the day prior to treatment. Each animal was treated with 5050 mg/kg of the test item. Each dose was moistened with a sufficient amount of DI water (1 ml/g test substance) and covered with a surgical gauze patch. The test substance was maintained in contact with the skin for 24 hours.

Observations for mortality and clinical/behavioural signs of toxicity were made at least three times on the day of dosing (Day 0) and at least once daily thereafter for 14 days. Individual body weights were recorded just prior to dosing and on Days 7 and 14.

Observations for evidence of dermal irritation were made at approximately 60 minutes after removal of wrappings and on Days 4, 7, 11 and 14.

On day 14, after dosing, animals were euthanised and subjected to gross necropsy and all anomalies recorded.

Findings

Mortality

There was no mortality during the study. The estimated acute dermal LD_{50} , as indicated by the data, was determined to be >5050 mg/kg body weight.

Clinical symptoms

All animals appeared normal for the duration of the study. There were no signs of dermal irritation at any observation during the study.

Body weight and body weight gain

The individual animals exhibited weekly weight gain during the study, with the exception of one female that lost 4g between Days 0 and 7.

Necropsy

Gross necropsy conducted at the termination of the study revealed no observable abnormalities.

Conclusions

The test substance, *B. bassiana* strain PPRI 5339, was evaluated for its acute dermal toxicity potential and relative skin irritancy when administered to albino rats. The acute dermal LD_{50} , as indicated by the data, is > 5050 mg/kg in males and females. No assessment of classification is necessary based on this study as Regulation (EC) No 1272/2008 does not apply to

microorganisms.

MA 5.2/05

Report: [REDACTED] (2007a) Acute dermal toxicity study of test item Bb spore concentrate in rats. [REDACTED]

[REDACTED]
Unpublished Report No. 06/338-002P

Guidelines: OECD Guidelines for Testing of Chemicals No.: 402
EU Method B.3
OPPTS 870.1200

GLP: Yes

Deviations: None

Aim of the Study

The acute dermal toxicity of test item Bb (*B. bassiana* strain PPRI 5339) spore concentrate was assessed in RL(WI) BR rats to provide information on health hazards likely to arise from 24 hour exposure by the dermal route.

Materials and methods

Day of Treatment: 01 February 2007

End of Experiment: 15 February 2007

Test Item

Name: Bb spore concentrate
Batch No.: F1096
Physical State: Uniform cream powder
Expiry Date: June 2007
Storage conditions: Store away from light in a cool (5 °C), dry dark place

CRL(WI)BR Wistar rats (provided by Charles River (Europe) Laboratories Inc., TOXI COOP Ltd., 1103 Budapest, Cserkesz u. 90) in acceptable health condition were used for the study.

Five male and five female animals were treated with Bb (*B. bassiana* strain PPRI 5339) spore concentrate by a single 24-hour dermal exposure in its original form at a dose level of 2000 mg/kg bw. 24 hours prior to the start of the treatment period the trunk of each animal was shaven. The test item was applied as a single dose uniformly over an area of approximately 10 per cent of the total body surface for a 24-hour exposure period. Sterile gauze pads were placed over the skin of each animal where the test item was applied. The gauzes were kept in contact with the skin by a patch with an adhesive hypoallergenic plaster.

Clinical examinations were made on the day of treatment 1 hour and 5 hours after the application of the test item, and once each day for 14 days thereafter. The body weights of animals were

recorded on day 0 and weekly thereafter. A gross necropsy was performed in all animals at the end of observation period.

Findings

Mortality

No mortality occurred during the study.

Clinical symptoms

There were no behavioural changes or general signs of toxicity in male or female animals treated at 2000 mg/kg bw.

Dermal alterations were observed in one female animal. Erythema and mild oedema were noted on the treated skin from day 1, which recovered between days 2 and 4 furthermore epithelization was registered between days 2 and 8.

Body weight and body weight gain

The body weight development was undisturbed in male and female animals during the 14 day observation period, i.e. similar to that expected in untreated animals of the same age and strain.

Necropsy

No macroscopic alterations related to the toxic effect of the test item were seen during the necropsy.

Conclusions

Under these experimental conditions, the acute dermal value of the test item Bb spore concentrate was greater than 2000 mg/kg in male and female CRL:(WI) BR rats. No assessment of classification is necessary based on this study as Regulation (EC) No 1272/2008 does not apply to micro-organisms.

MA 5.2.1 Sensitisation

Following the PRAPeR M2 meeting on 16-18th February 2009 in Parma Italy (Doc K-MA 5.2.1/01), a skin sensitisation study is no longer required for a European registration of a microorganism active substance. All microorganisms are considered to be potential allergens and the phrase 'may invoke a sensitising reaction' must appear on the label. This was also proposed by the Advisory Committee on Pesticides (ACP) of the UK at its meeting held on May 2012 (Doc K-MA 5.2.1/02)

A single study on the skin sensitisation of *B. bassiana* is submitted by the registrant and is summarised in the following table.

Table MA 5.2.1-1: Summary of sensitisation studies submitted for *Beauveria bassiana* strain PPRI 5539

M-MA Point	Study/Route/ Method	Species	Dose per Animal	Results	Conclusion	Reference
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5.2.1/03	Skin Sensitisation OECD 406	Guinea Pigs	0.01% (w/v) intra-dermal, 20% (w/v) dermal induction. 5% (w/v) challenge	Positive	Sensitising	(2007b)
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Severe autoimmune reactions were seen in the lungs of rats during acute inhalation toxicity studies submitted under Point M-MA 5.2.2.2 when exposure was conducted using aerosols generated from the powdered test item. Histopathological investigation of the lungs of rats exposed to a 5.39 mg/L aerosol of the test item for 4 hours revealed moderate to severe lesions, including necrosis of the lining of the epithelium of secondary bronchi, terminal bronchioles and alveolar ducts. These effects were also accompanied by acute inflammation of the walls of these airways that extended to adjacent alveoli in one animal. Many of these adjacent alveoli contained free inflammatory cells and cellular debris. There was diffuse congestion of blood vessels and alveolar walls, and many free blood cells (hemorrhage) in alveoli. The effects seen in the acute inhalation studies are consistent with an allergic reaction to aerosol exposure and mortality was believed to have been caused by asphyxiation due to inflammation of the lungs. The build up of cellular debris may have also contributed to asphyxiation. See point M-MA 5.2.2.2 for further information.

MA 5.2.1/03

Report: (2007b) Skin sensitisation of Bb spore concentrate in Guinea Pigs by Magnusson and Kligman. Unpublished Report No. 06/338-104T.

Guidelines: OECD Guidelines for the Testing of Chemicals No.: 406

GLP: Yes

Deviations: The batch number of sunflower oil used was different to that outlined in the study plan. This deviation did not affect the validity of the study.

Aim of the Study

The aim of the study was to evaluate the skin sensitisation potential of Bb (*B. bassiana* strain PPRI 5339) spore concentrate in the guinea pig using the Magnusson and Kligman method.

Materials and methods

Start of Experiment: 27 February 2007

End of Experiment: 23 March 2007

Test Item

Name: Bb spore concentrate

Batch No.:	F1096
Physical State:	Powder
Colour:	Uniform cream powder
Purity:	Not reported
Storage conditions:	In a cool (5°C), dry and dark place.

Before the preliminary dose range finding study, a test item formulation evaluation was performed which concluded that a concentration of 30 % (in vehicle) was the maximum practical formulation that would be prepared and practically applied and that intradermal concentrations should be ≤ 1 %.

Male Dunkin Hartley guinea pigs (provided by LAB-ÁLL Bt. Budapest, 1174 Hunyadi u. 7) in acceptable health condition were used for the study. Before exposure, an area of approximately 5 x 5 cm on the scapular region was clipped free of hair and shaven close with care.

Following a preliminary dose range finding study, intra-dermal treatment the test item was used at concentration of 0.01 % (w/v). Dermal induction treatment was conducted at a concentration of 20 % (w/v). For the challenge exposure, all animals of the treatment and control group were treated with concentrations of 5 % (w/v) test item in vehicle. The time of exposure was 24 hours (for the intra-dermal and challenge doses) and 48 hours (for the dermal induction dose). Symptoms were examined and scored 1, 24, 48 and 72 hours after exposure.

For intra-dermal induction row of 3 injections (6 in all) was made on each side of the test animal. Two injections were made with 0.1 mL of Freund's complete adjuvant (FCA) mixed with physiological saline solution (1:1). Two injections were made with 0.1 ml of the test item (0.01 %) homogenized in vehicle (95% sunflower oil and 5 % cremophor). The final two injections were made with 0.1 ml of test item (0.01 %) mixed with physiological saline solution and homogenized in Freund's complete adjuvant (1:1). The control animals were treated similarly as the test group but the vehicle, without the test item, was used for injections.

For dermal induction, seven days after the intra-dermal induction, 0.5 mL of the test item (20 %, w/v) was spread on the surface prepared previously and covered with a standard (5x5 cm) porous gauze patch for 48 hours. Control animals were treated with vehicle only.

For the challenge exposure, animals were exposed using a closed patch. Left shaved flank areas of the animals (both the test and the control) were treated with 0.5 ml of the test item (at concentration of 5 %). The right shaved flank areas were treated with 0.5 ml of vehicle (95% sunflower oil and 5 % cremophor), in all cases.

Dermal irritation scores were evaluated in accordance with the scoring system by Draize (1959).

The main study design is summarised in the following table.

Table MA 5.2.1/03-1: Study design used in main skin sensitisation test.

	Intra-dermal Induction Exposure (Main Study I)		Dermal Induction Exposure (Main Study II)		Challenge Exposure (Main Study III)	
	Control	Test	Control	Test	Control	Test
Dose Group	Control	Test	Control	Test	Control	Test
No. of animals	5	10	5	10	5	10
Test Item	-	0.01	-	20	5	5

Concentration (%)						
Day of Treatment	1	1	8	8	22	22
Time of Exposure (h)	24	24	48	48	24	24
Time of Observations	24 hours after the treatment	24 hours after the treatment	1, 24, 48 and 72 hours after the patch removal	1, 24, 48 and 72 hours after the patch removal	24 and 48 hours after the patch removal	24 and 48 hours after the patch removal

Findings

Test Group

A positive response was seen in eight out of ten animals in the test group (80 %). The mean Draize response scores were 1.6 and 1.2 at 24 and 48 hours respectively. The dermal scores observed on the skin surface of previously sensitised guinea pigs represented patchy to confluent erythema. On the opposite (right) side treated with vehicle no reaction was found.

The dermal responses observed following challenge with the test item in the main study are presented in the following tables.

Table MA 5.2.1/03-2: Dermal symptoms of guinea pigs challenged with the test item (test group)

Animal Number Sex: male	Skin Reaction			
	24 hours (after patch removal)		48 hours (after patch removal)	
	Left side (test item)	Right side (vehicle only)	Left side (test item)	Right side (vehicle only)
104	confluent erythema	n.v.c	patchy erythema	n.v.c
106	confluent erythema	n.v.c	patchy erythema	n.v.c
107	confluent erythema	n.v.c	confluent erythema	n.v.c
108	n.v.c	n.v.c	n.v.c	n.v.c
109	confluent erythema	n.v.c	confluent erythema	n.v.c
111	confluent erythema	n.v.c	patchy erythema	n.v.c
112	n.v.c	n.v.c	n.v.c	n.v.c
113	confluent erythema	n.v.c	confluent erythema	n.v.c
114	confluent erythema	n.v.c	confluent erythema	n.v.c
115	confluent erythema	n.v.c	patchy erythema	n.v.c

Table MA 5.2.1/03-3: Dermal response of guinea pigs challenged with the test item (test group)

Animal Number Sex: male	Scores of Dermal Reaction	
	24 hours (after patch removal)	24 hours (after patch removal)
104	2	1
106	2	1
107	2	2
108	0	0
109	2	2
111	2	1
112	0	0
113	2	2
114	2	2
115	2	1
Mean of Scores	1.60	1.20
% Positive	80	80

Control Group

No visible changes to the skin of the control animals was observed throughout the study.

Body Weight

There were no notable differences between the test animal group and the control group.

Conclusions

The results of the study indicate that Bb concentrate is a skin sensitiser. Whilst classification under Regulation (EC) No 1272/2008 is not necessary for microorganisms, all microorganisms are considered to be potential allergens and the phrase 'may invoke a sensitising reaction' must appear on the label.

MA 5.2.2 Acute toxicity, pathogenicity and infectiveness

MA 5.2.2.1 Acute oral toxicity, pathogenicity and infectiveness

A summary of acute oral toxicity, pathogenicity and infectiveness studies submitted by the registrant is presented in the following table. The data submitted by the registrant indicates that *B. bassiana* is not acutely toxic *via* the oral route, nor is there any evidence of pathogenicity or infectiveness.

Table MA 5.2.2.1-1: Summary of acute oral toxicity, pathogenicity and infectiveness for *Beauveria bassiana* strain PPRI 5539

M-MA Point	Study/Route/Method	Species	Dose per Animal	Results	Conclusion	Reference
5.2.2.1/01	Acute Oral Toxicity OECD 425	Rat	5000 mg/kg	No mortality observed during study No other significant observations	= >5000 mg/kg	(2011d)
5.2.2.1/02	Acute Oral Toxicity OECD 423	Rat	2000 mg/kg	No mortality observed during study No other significant observations	= >2000 mg/kg	(2007b)
5.2.2.1/03	Oral pathogenicity following exposure via feed	Mouse	Conidia suspension of 1 x conidia/mL added to animal's feed	No evidence of pathogenicity or other pathological changes. Clearance of conidia in faeces after 7 days.	Not pathogenic	Acosta, ME <i>et al.</i> (2012)

Acute oral toxicity, pathogenicity and infectivity studies summarised in the DAR for *B. bassiana* strain ATCC and also indicate no mortality or evidence of pathogenicity or infectiveness. *B. bassiana* strain GHA showed clearance of the microorganism 3 days after exposure. In strain ATCC 74040 *B. bassiana* was not considered to be pathogenic, although colonies were still observed in the faeces 14 days after dosing.

Similarly, Zimmerman (2007)³ presented a summary of existing publicly available literature on studies with laboratory animals at the time indicating no evidence of acute oral toxicity, pathogenicity or infectivity with acute oral values quoted as >1.8 x CFU/kg (strain ATCC 74040), >5000 mg/kg (strain IMBST 95.031) and >1.1 x CFU/kg (strain IMBST 95.041).

MA 5.2.2.1/01

Report: (2011d), Acute oral toxicity study (UDP) in rats.

Unpublished Report No. 15367-11

Guidelines: Guidelines for the Testing of Chemicals No.: 425.

US EPA OCSP 870.1100

FIFRA 7 USC 136, OECD

GLP: Yes

Deviations: The provided Certificate of Analysis was not accompanied by a GLP compliance statement.

Stability information was not provided to the testing facility.

Mixture analysis was not performed.

Relative humidity was at times outside protocol range.

The deviations listed did not affect the outcome of the study.

Aim of the study

The objective of this study was to assess the acute oral toxicity potential of the test substance when administered by gavage to rats.

Materials and methods

Day of Treatment: 23 August 2011

End of Experiment: 15 September 2011

Test Item

Name: *Beauveria bassiana* strain PPRI 5339

Batch No.: S627

Physical State: Light cream powder

Purity: 2.06 x spores/g provided information

Storage conditions: Room temperature

Albino Sprague-Dawley rats (provided by Texas Animal Specialities, Humble, TX) in acceptable health condition were used for the study.

Three animals (all female) were selected for the study. The test substance was mixed with water to produce a 40% w/v concentration. An individual dose was calculated based on its fasted body weight and administered by gavage at a volume of 12.5 mL/kg. Each dose was administered using an appropriately sized syringe and stainless steel ball-tipped intubation needle.

Observations for mortality and clinical/behavioural signs of toxicity were made at least three times on the day of dosing (Day 0) and at least once daily thereafter for 14 days. Individual body weights were recorded just prior to dosing and on Day 7 and 14.

On day 14, after dosing, each animal was euthanized by an overdose of . All study animals were subjected to gross necropsy and all abnormalities were recorded.

Findings

Mortality

There was no mortality during the study. The estimated acute oral , as indicated by the data, was determined to be greater than 5000 mg/kg.

Clinical symptoms

The only clinical sign presented was activity decrease in one animal on the day of dosing.

Body weight and body weight gain

All individual animals exhibited weekly weight gain during the study.

Necropsy

Gross necropsy conducted at the termination of the study revealed no observable abnormalities.

Conclusions

The test substance, *B. bassiana* strain PPRI 5339, was evaluated for its acute oral toxicity potential when administered to albino rats. The acute oral LD_{50} is estimated to be >5000 mg/kg in females.

MA 5.2.2.1/02

Report: [REDACTED] (2007b), Acute oral toxicity study (acute toxic class method) of test item Bb spore concentrate in rats. [REDACTED]

Unpublished Report No. 06/338-001P

Guidelines: OECD Guidelines for the Testing of Chemicals No.: 423
EU Method B.1.
US EPA OPPTS 870.1100

GLP: Yes

Deviations: None

Aim of the Study

An acute oral toxicity study was performed to assess the acute toxicity of test item Bb (*B. bassiana* strain PPRI 5339) spore concentrate in rats. The results of the study allowed the test item to be ranked according to the classification systems, currently in use.

Materials and methods

Day of Treatment: 01 and 02 February 2007

End of Experiment: 15 and 16 February 2007

Test Item

Name: Bb spore concentrate
Batch No.: F1096
Physical State: Uniform cream powder

Expiry Date: June 2007
Storage conditions: Store away from light in a cool (5 °C), dry dark place

Female CRL(WI)BR Wistar rats (provided by Charles River (Europe) Laboratories Inc., TOXI COOP Ltd., 1103 Budapest, Cserkesz u. 90) in acceptable health condition were used for the study.

A single oral treatment was carried out by gavage for each animal after an overnight food withdrawal. The animals were treated at a dose level of 2000 mg/kg bw with a Bb spore concentrate of 200 mg/mL prepared with mixture of 95% (v/v) sunflower oil and 5% (v/v) cremophor. The dose rate corresponded to a treatment volume of 10 mL/kg bw.

Clinical observations were performed for all animals 30 minutes, 1, 2, 3, 4 and 6 hours after dosing and daily for 14 days thereafter. Food was made available again 3 hours after the treatment. Body weight was measured weekly. Gross necropsy was performed on all animals at termination of examination (day 14).

Results

Mortality

No mortality occurred after a single oral administration of Bb spore concentrate at 2000 mg/kg bw dose level in female CRL:(WI) BR rats.

Clinical symptoms

No animals showed clinical symptoms on the day of the treatment and the following 14-day observation period in either groups. The physical condition and behaviour of animals were normal in all animals during the following 14-day observation period.

Body weight and body weight gain

The mean body weight and the body weight gain of animals were in the normal range during the two weeks observation period, similar to the expected values in untreated animals of the same age and strain.

Necropsy

No macroscopic alterations related to the toxic effect Bb spore concentrate were found.

Conclusions

The acute oral LD₅₀ value of the test item Bb spore concentrate was > 2000 mg/kg bw in female CRL:(WI) BR rats.

MA 5.2.2.1/03

Reference:	Acosta, ME, Perfetti, DC, Eduarte, G, Moreno, PM (2012) Oral pathogenicity of an autochthonous isolate of the entomopathogenic fungus <i>Beauveria bassiana</i> in mice (<i>Mus musculus</i> , strain NMRI). Boletín de Malariología Salud Ambiental, (2012) 52(1): 79-86
Guidelines:	None
GLP:	Not applicable

Summary

This study evaluated the pathogenicity of native isolate LF14 of *B. bassiana* (Fungi: Ascomycota) following oral administration to white mice (NMRI strain), both male and female (12 animals per sex). A suspension of 1 x conidia/mL was added to the food of the animals (amount not specified) and was consumed for five days (food and treatment was renewed daily). Control animals were fed on diet treated with only distilled water. Daily observations included clinical behaviour, body weight and assessment of physiological clearance of *B. bassiana* by analysis of faeces. Clinical observations included signs of irritation in skin, fur, eyes and mucous membranes, overall assessment of health and behaviour. For clearance, direct examination of faeces were made mounting faecal samples on slides and staining cotton blue and viewing under a light microscope. Faecal samples were also cultured on solid medium for 7-14 days at 26 °C and >90 % relative humidity. The number of germinated conidia (%) was estimated after 24, 48 and 72 hours. Following the incubation period sporulation (conidia/mL) was assessed using a haemocytometer.

Each animal was subject to complete necropsy on days 3, 7 and 14 after treatment, including infectivity analysis of sampled tissues, which were cultured on solid medium. For histopathology, a section of the tissue samples were fixed in 10% formaldehyde and embedded in paraffin for histological sections 5-7 µm. The sections were stained with haematoxylin - eosin (H & E) and evaluated and photographed under a light microscope. Organs assessed were skin, kidney, liver, lung, spleen, stomach, small bowel, intestine heart and trachea.

Statistical analysis was conducted to determine any significant differences between mean body weights, % germination of conidia and sporulation. Analysis was conducted using the statistical package MINTIAB version 13.2.

Animals showed no discernible pathological changes or death and had 100% active-normal behaviour. In all the mice groups, including control ones, there was an increase in the mean weight gained through time, with statistically significance difference between the sexes ($p < 0.0000$). Viable conidia were observed in faeces until 7 days after consuming *B. bassiana*. No anatomopathological changes or germinated conidia were detected in analysed organs, with normal tissue reactions, suggesting no evidence for fungal multiplication.

Conclusions

On the bases of this study it was concluded that isolate LF14 of *B. bassiana* orally administered is non pathogenic in white mice (NMRI strain).

MA 5.2.2.2 Acute inhalation toxicity, pathogenicity and infectiveness

A summary of acute inhalation toxicity, pathogenicity and infectiveness studies submitted by the registrant is presented in the following table.

Table MA 5.2.2.2-1: Summary of acute inhalation toxicity, pathogenicity and infectiveness for *Beauveria bassiana* strain PPRI 5539

M-MA Point	Study/Route /Method	Species	Dose per Animal	Results	Conclusion	Reference
5.2.2.2/01	Acute Pulmonary Toxicity/ Pathogenicity	Rat	7.7 x CFU/rat	A single animal died during the study. No other signs of toxicity or pathogenicity	Non-toxic and non-pathogenic to rats	██████████ (2013)
5.2.2.2/02	Acute Inhalation Toxicity	Rat	5.39 mg/L (aerosol) for 4 hours	Animals dosed were found dead on Day 2 of the study	= <5.39 mg/L	██████████ (2012a)
5.2.2.2/03	Acute Inhalation Toxicity OECD 403	Rat	2.59 & 5.28 mg/L (aerosol) for 4 hours	Mortality by day 14: 6/10 for 5.28 mg/L 7/10 for 2.59 mg/L	= <2.59 mg/L	██████████ (2012)
5.2.2.2/04	Histopathological evaluation of the lungs from both animals used in ██████████, 2012a	Rat	N/A	Lesions – necrosis of epithelial lining. acute inflammation, alveolar cellular debris, haemorrhaging in all lobes.	Mortality in ██████████ (2012a) was due to local effects of inflammation and not systemic toxicity	██████████ (2011)

Acute inhalation studies by ██████████, 2012a & 2012b (see MA 5.2.2.2/01 & 5.2.2.2/02) resulted in 100 % mortality following 4 hour aerosol exposure to *B. bassiana* at dose levels of ≥ 2.59 mg/L. ██████████, 2012a exposed two male rats to an aerosol concentration of 5.39 mg/L, resulting in both animals being found dead on day 2 of the study. One day after exposure, sub-lethal effects included piloerection, a decrease in activity, a respiratory gurgle and body tremors. Gross necropsy revealed red crust around the mouth and mottled lungs and liver.

██████████ 2011 (see MA 5.2.2.2/03) conducted histopathological evaluation of the lungs used in ██████████, 2012a. Both sets of lungs contained moderate to severe lesions, including necrosis of the lining of the epithelium of secondary bronchi, terminal bronchioles and alveolar ducts. These effects were also accompanied by acute inflammation of the walls of these airways that extended to adjacent alveoli in one animal. Many of these adjacent alveoli contained free

inflammatory cells and cellular debris. There was diffuse congestion of blood vessels and alveolar walls, and many free blood cells (hemorrhage) in alveoli. The lesions were present throughout the entire lobe with essentially the same severity in all lobes submitted.

█, 2012b exposed 10 rats (5 male and 5 female) each at concentrations of 5.28 mg/L and 2.59 mg/L. After 14 days post exposure, 6/10 rats were dead at 5.28 mg/L and 7/10 rats were dead at 2.59 mg/L. Sub-lethal effects were limited to 3 days post-exposure and consisted of piloerection, a decrease in activity and a respiratory gurgle. Gross necropsy findings on animals that died during the study consisted of crusted muzzle, strained/matted genital fur, discoloured lungs/liver/kidneys and empty gastrointestinal tract. Gross necropsy on the seven animals surviving to termination of the study revealed no observable abnormalities except discoloured lungs in one male and two females. No histopathological assessment on the lungs was conducted for this study.

The submitted studies are in contrast to acute inhalation toxicity studies conducted for other strains of *B. bassiana* (ATCC and), where no mortality was observed, but local effects of inflammation/immune reaction and organ weight increase were observed in the lungs in addition to accumulation of cellular debris. These studies were conducted using an intratracheal application method, where the test item is applied directly onto the trachea.

The mortality seen in studies by █, 2012a & 2012b, is considered to be an artefact of the test method employed, i.e. aerosol exposure for a period of 4 hours. Such an exposure regime would induce a much more extreme inflammation/immune reaction than that seen in the intratracheal exposure, due to the sheer amount of the test item filling the lungs for 4 hours. Build up of cellular debris may also have contributed to the observed mortality in the 4 hour exposure. The pathological observations in the lungs support asphyxiation as a cause of death in the animals due to inflammation and necrosis as a result of an autoimmune reaction in the lung tissue.

Microorganisms are generally thought to have the potential to provoke sensitising reactions. The primary and supporting information submitted indicate that *B. bassiana* has the potential to induce inhalatory allergenicity and allergic skin reactions in laboratory animals and humans. Data from other *Beauveria bassiana* strains (ATCC and) indicate that this effect is not strain specific and is a mechanism for the species in general. When *B. bassiana* strain PPRI 5339 was tested with a similar acute intratracheal challenge (█ 2013) as reported for strains ATCC and , similar results to that of ATCC 74040 and GHA were seen with no test animal mortality as a result of treatment with active PPRI 5339.

After an acute intratracheal challenge of *B. bassiana* strain of 5.7 mg/kg in males and 6.7 mg/kg in females (viable and killed spores), there were no unscheduled deaths in this study, but the absolute and relative weight of the lungs and associated lymph nodes were markedly higher in the groups receiving viable and killed spores. The increase in lung weight was in line with histopathological findings. By day 3, the pathological changes in both sexes had progressed to perivascular inflammation involving different cell types with macrophages, lymphocytes and plasma cells being predominant. Furthermore, hyperplasia of lymphoid tissue and alveolar oedema were observed. Incidence and severity of the lesions appeared higher in the group treated with killed spores than in the viable spores group. Inflammation and lymphoid tissue hyperplasia were present in similar severity scores on day 7, too, but the inflammatory changes tended to change at that time suggesting microgranuloma formation. Evidence of the presence of viable fungus such as mycelia was not found at any time. Microbial clearance was completed by

day 7 since, in both sexes, the detection of viable spores in the TS group was confined to lungs and associated lymph nodes on days 0 and 3. The microbial count showed a clear decline by one (males) to three orders of magnitude (females) during that interval. Thus, there was no spread of the microorganism to other parts of the body or to the shelf control group that was housed together with the viable spore animals in the same room. No test substance could be recovered from the killed spore or control groups. The study concluded that *B. bassiana* strain GHA was not infective and had an acute intratracheal of > 5.7 mg/kg.

After an acute intratracheal challenge of *B. bassiana* strain ATCC 74040 of 2.5 x CFU in 0.04 mL of saline to 57 rats per sex, there were no unscheduled deaths in this study, but at necropsy tan or light brown lesions in all lobes of the lungs of most treated animals were noted. At histopathological examination at the earlier necropsies, lungs exhibited marked multifocal subacute to chronic inflammation. The presence of significant numbers of macrophages was noted in animals sacrificed at later time points. However, little destruction of the lung parenchyma was noted. No lung findings were reported on day 22 and afterwards. No colonies were detected in any of the tissue homogenates other than lungs with the exception of one kidney sample from a treated male on day 4 and one treated female liver collected on day 15. From day 15 onwards the test microorganism was not found in the lung samples. Control animals did not show any clinical signs of toxicity or gross necropsy findings. The test microorganism was not found in any tissue sample. The study concluded that *B. bassiana* induced reversible signs of toxicity and that the substance was not pathogenic or infectious.

No further studies on acute inhalation toxicity are considered necessary for this endpoint and inhalatory allergenicity is assumed for *B. bassiana* strain PPRI 5339. The label for this substance must include the phrase "Microorganisms may have the potential to provoke sensitising reactions".

MA 5.2.2.2/01

Report: [REDACTED], A, (2013), *Beauveria bassiana* strain PPRI 5339 Acute Pulmonary Toxicity/Pathogenicity in Rats. [REDACTED]
[REDACTED]
Unpublished Report No. 16701-12

Guidelines: None

GLP: Yes

Aim of the study

The aim of the study was to assess the acute toxicity and pathogenicity of *Beauveria bassiana* strain PPRI 5339 following intratracheal exposure to the rat.

Materials and methods

Test Item

Name: *Beauveria bassiana* strain PPRI 5339 Spore Concentrate
Batch No.: S627
Physical State: Light cream powder

Purity: 2.06 x spores/g provided information
Storage conditions: Refrigerated

Albino Sprague Dawley rats (provided by Texas Animal Specialities, Humble, TX) in acceptable health condition were used for the study.

Thirty six male and 36 female rats (nulliparous and not pregnant) plus three validation animals were selected for the study. The three validation animals were used to assess recovery efficiency from test animals during the study.

A 1g sample of the test item was serially diluted with sterile phosphate buffer saline (PBS). A portion of 12 mL was rendered non viable through autoclaving for 45 min. The test substance, or inactivated test substance, was administered into the trachea of each animal in a single 0.1 mL dose of the dilution prepared in PBS. A group of 6 animals per sex were used for the untreated control (Group I), 4 animals per sex for the shelf control (Group II), 8 animals per sex for the inactive test item (Group III) and 18 animals per sex for the viable test item.

Body weights were recorded pre-dose on day 0 and weekly thereafter. Observations for mortality and signs of pharmacological and/or toxicological effects were made three times following dosing and daily thereafter for 21 days. Observations included, but were not limited to the skin, fur, eyes and mucous membranes; respiratory circulatory, autonomic and central nervous systems; somatomotor activity and behaviour patterns. Particular attention was given to tremors, convulsions, diarrhea, lethargy, salivation, sleep and coma.

On Day 0, three animals per sex from the viable test item group were sacrificed, and 3 animals per sex were sacrificed weekly thereafter. Two animals per sex from the untreated control, 1 animal per sex from the shelf control and three animals per sex from the inactive test item were sacrificed on Day 0 after dosing. All other animals were sacrificed at study termination. A gross necropsy was conducted on each animal at the interim time of sacrifice or at the time of discovery of death. The gross necropsy included gross observations of external surfaces, all orifices and thoracic, abdominal and pelvic cavities. Organ weights were recorded for all animals sacrificed.

Quantification of the test microbe was performed on tissues removed at each interim sacrifice to determine the infectivity/pathogenicity and estimate clearance of the test item after dosing. On Day 0, lungs were the only tissues assessed. At other time points, tissues/fluid removed from each animal included: kidneys, brain, liver, lungs, spleen, blood, representative lymph nodes and cecum contents. Histopathological evaluation was only conducted on abnormal tissues. All tissues and fluid samples removed for quantification were cultured to provide qualitative and quantitative measurements of the test microbe.

One-way parametric analysis of variance (ANOVA) was performed on the recorded body weights, body weight change and relative organ weights as a percentage of body weights.

Findings

The only mortality during the study was a single animal from the inactive test item group on Day 19. No abnormalities were noted during clinical observations. No observable abnormalities

were noted except in the animal that was found dead from the non-viable test item group, in this animal the organs were black. Clearance of test item from the lungs was achieved by day 14 as quantification results from plated tissues yielded two consecutive counts that were below the clearance threshold. The test item was not observed in any blood, brain, lungs, liver, spleen, kidneys, mesenteric lymph nodes and cecum contents by Day 7. A summary of the quantification of test item in observed tissues is presented in the following table.

Table MA 5.2.2.2/01-1: Summary of quantification of organisms in tissues

	Blood CFU/mL	Brain CFU/g	Lungs CFU/g	Liver CFU/g	Spleen CFU/g	Kidneys CFU/g	MLN CFU/g	Cecum Contents CFU/g
Day 0	NA	NA	0	NA	NA	NA	NA	NA
Day 3	0/<100	0/<100	1.1 x	0/<100	0/<100	0/<100	9.4 x	0
Day 7	0	0	0	0	0/<100	0	0	0
Day 14	0	0	0	0	0	0	0	0

NA – Not applicable, only lungs were collected from animals sacrificed on Day 0

MLN – Mesenteric Lymph Nodes

0/<100 – all values were either less than 100 or 0

Conclusion

With no plated organisms seen and no observed abnormalities during general health observations at necropsy, there is no evidence for toxic or pathogenic effects caused by the test item. The test item was concluded to be non-toxic to rats when administered by tracheal injection in a single dose of 7.7 x CFU/mL or 7.7 x CFU/rat.

MA 5.2.2.2/02

Report: [REDACTED] (2012a), Acute inhalation toxicity in rats. [REDACTED]

Unpublished Report No. 15687-11

Guidelines: None

GLP: No

Aim of the study

The objective of this study was to evaluate the test substance for its acute inhalation toxicity potential in male albino rats (Sprague Dawley).

Materials and methods

Day of Treatment: 23 August 2011

End of Experiment: 15 September 2011

Test Item

Name: *Beauveria bassiana* strain PPRI 5339

Batch No.: S627

Physical State: Light cream powder
 Purity: 2.06 x spores/g provided information
 Storage conditions: Room temperature

Albino Sprague-Dawley rats (provided by Texas Animal Specialities, Humble, TX) in acceptable health condition were used for the study.

Two male rats were selected for testing and were exposed to an aerosol generated from the undiluted test substance (fine powder) for a period of four hours. When 99% concentration (t-99) was attained, the animals were inserted into a ~ 15 L stained steel nose-only inhalation chamber for the specified exposure period. At the termination of the exposure period, the animals were returned to their stock laboratory cages.

The concentration of the test substance in the exposure atmosphere was determined gravimetrically twice per hour and nominally at the end of the exposure. The nominal concentration was determined by dividing the loss in weight of the test substance after the exposure by the total volume of air passed through the chamber.

Particle size was determined twice during the exposure, using a cascade impactor. The MMAD and particle size distributions are calculated from these data using probit analysis.

Observation for mortality and signs of pharmacological and/or toxicological effects were made frequently on the day of exposure (Day 0) and at least once daily thereafter. Individual body weights were recorded just prior to the inhalation exposure and at the time of discovery after death.

Both animals were subjected to gross necropsy and all normalities recorded. Sections of each lung of both animals were preserved in 10% neutral buffered formalin for histopathological examination by Colorado Histo-Prep (see CA 5.2.2.2/03).

Results

Mortality/Estimated values

Both animals were found on Day 2 of the study. The acute inhalation for *Beauveria bassiana* is < 5.39 mg/L in male albino rats.

Body weights

Individual body weights are presented in the following table

Table MA 5.2.2.2/02-1: Body weights, time of death and gross necropsy findings for acute inhalation toxicity of *Beauveria bassiana* strain PPRI 5339 in rats

Animal Number	Body Weights (g)			Time of Death	Gross Necropsy Findings
	Day 0	Day 7	Final		
31-M	270	-	234	Day 2	Red crust around mouth; lungs & liver mottled
32-M	262	-	228	Day 2	Red crust around mouth; lungs & liver mottled

Indicates time of discovery after death

Pharmacologic and/or Toxicological Signs

All observations are presented in the following tables.

Table MA 5.2.2.2/02-2: Pharmacologic and /or toxicologic signs in acute inhalation toxicity study of *Beauveria bassiana* in albino rats up to 6 hours after exposure.

Reaction and severity	Hours after exposure begins				
	0.5	1.0	2.5	4.5	6.0
Piloerection (m)	0	0	0	2	2
Activity increase (m)	0	0	0	3	3

v – very slight

s – slight

m – moderate

e – extreme

Note: Digits indicate number of animals exhibiting reaction.

Table MA 5.2.2.2/02-3: Pharmacological and /or toxicological signs in acute inhalation toxicity study of *Beauveria bassiana* in albino rats up to 14 days after exposure.

Reaction and severity	Days after exposure	
	1	2
Piloerection (e)	2	
Activity decrease (e)	2	
Respiratory gurgle	2	
Body tremors	2	
Death	0	2

v – very slight

s – slight

m – moderate

e – extreme

Note: Digits indicate number of animals exhibiting reaction. If discovery was between scheduled observations, death is presented under next observation time.

Necropsy findings

Individual necropsy findings are presented in Table 5.2.2.2/01-1. The gross necropsy conducted on each animal revealed abnormal findings of red rust at the mouth and mottled lungs and liver.

Conclusion

Beauveria bassiana was evaluated for its acute inhalation toxicity potential in albino rats. The acute inhalation LD_{50} is < 5.39 mg/L in males.

CA 5.2.2.2/03

Report:

(2012b), Acute inhalation toxicity study in rats.

Unpublished Report No. 15369-11

Guidelines:

OECD Guidelines for the Testing of Chemicals No.: 403

US EPA OCSP 870.1300

FIFRA 7 USC 136

GLP:	Yes
Deviations:	The provided Certificate of Analysis was not accompanied by a GLP compliance statement. Stability information was not provided to the testing facility. Relative humidity was at times outside protocol range. The deviations listed did not affect the outcome of the study.

Aim of the study

The objective of this study was to assess the acute inhalation toxicity potential of the test substance in albino rats.

Materials and methods

Day of Treatment: 18 August and 14 September 2011

End of Experiment: 1 September and 28 September 2011

Test Item

Name:	<i>Beauveria bassiana</i> strain PPRI 5339
Batch No.:	5627 C2
Physical State:	Light cream powder
Purity:	2.06 x spores/g provided information
Storage conditions:	Room temperature

Albino Sprague-Dawley rats (provided by Texas Animal Specialities, Humble, TX) in acceptable health condition were used for the study.

Twenty rats (5 male and 5 female per each of two exposure levels) were selected for testing and were exposed to an aerosol generated from the undiluted test substance (fine powder) for a period of four hours at concentrations of either 5.28 mg/L or 2.59 mg/L. When 99% concentration (t-99) was attained, the animals were inserted into an ~ 15 L stained steel nose-only inhalation chamber for the specified exposure period. At the termination of the exposure period, the animals were returned to their stock laboratory cages.

The concentration of the test substance in the exposure atmosphere was determined gravimetrically twice per hour and nominally at the end of the exposure. The nominal concentration was determined by dividing the loss in weight of the test substance after the exposure by the total volume of air passed through the chamber.

Particle size was determined twice during the exposure, using a cascade impactor. The MMAD and particle size distributions are calculated from these data using probit analysis.

Observation for mortality and signs of pharmacological and/or toxicological effects were made frequently on the day of exposure (Day 0) and at least once daily thereafter for 14 days.

Individual body weights were recorded just prior to the inhalation exposure and at Days 7 and 14, or at the time of discovery after death.

On Day 14 after exposure, each surviving animal was euthanized. All study animals, whether dying during the study or euthanized were subjected to gross necropsy and all abnormalities recorded.

Results

Mortality/estimated values

Mortality data is presented in the following table.

Table MA 5.2.2.2/03-1: Mortality data for acute inhalation study of *Beauveria bassiana* on albino rats

Test Substance Conc. (mg/L)	Number dead/Number treated		
	Males	Females	Combination
5.28	3/5	3/5	6/10
2.59	4/5	3/5	7/10

The acute inhalation of *Beauveria bassiana* strain PPRI 5339 is < 2.59 mg/L to albino rats.

Body weights

Animals surviving to termination exhibited weekly weight gain during the study except in one male and one female that lost weight between Days 0 and 7.

Clinical signs

Clinical signs are presented in the following tables. Prominent in-life observations included activity decrease and piloerection, which were clear in surviving animals by Day 3. Respiratory gurgle was only observed in animals on test.

Table MA 5.2.2.2/03-2: Pharmacological and /or toxicological signs in acute inhalation toxicity study of *Beauveria bassiana* in albino rats up to 6 hours after exposure at a concentration of 5.28 mg/L.

Reaction and severity	Hours after exposure begins				
	0.5	1.0	2.5	4.5	6.0
Males					
Piloerection (m)	0	0	0	5	5
Activity increase (m)	0	0	0	5	5
Females					
Piloerection (m)	0	0	0	5	5
Activity increase (m)	0	0	0	5	5

v – very slight

s – slight

m – moderate

e – extreme

Note: Digits indicate number of animals exhibiting reaction.

Table MA 5.2.2.2/03-3: Pharmacological and /or toxicological signs in acute inhalation toxicity study of *Beauveria bassiana* in albino rats up to 14 days after exposure at a concentration of 5.28 mg/L.

Reaction and severity	Days after exposure													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Males														
Piloerection (m)	5	3	0	0	0	0	0	0	0	0	0	0	0	0
Activity decrease (m)	5	3	0	0	0	0	0	0	0	0	0	0	0	0
Death	0	2	1	0	0	0	0	0	0	0	0	0	0	0
Females														
Piloerection (me)	5	4	0	0	0	0	0	0	0	0	0	0	0	0
Activity decrease (m)	5	4	0	0	0	0	0	0	0	0	0	0	0	0
Death	0	1	2	0	0	0	0	0	0	0	0	0	0	0

v – very slight

s – slight

m – moderate

e – extreme

Note: Digits indicate number of animals exhibiting reaction. If discovery was between scheduled observations, death is presented under next observation time. Time of death indicates time of discovery after death. If discovery was between scheduled observations, death is presented under next observation time.

Table MA 5.2.2.2/03-4: Pharmacological and /or toxicological signs in acute inhalation toxicity study of *Beauveria bassiana* in albino rats up to 6 hours after exposure at an exposure concentration of 2.59 mg/L.

Reaction and severity	Hours after exposure begins				
	0.5	1.0	2.5	4.5	6.0
Males					
Piloerection (m)	0	0	0	5	5
Activity increase (m)	0	0	0	5	5
Females					
Piloerection (m)	0	0	0	5	5
Activity increase (m)	0	0	0	5	5

v – very slight

s – slight

m – moderate

e – extreme

Note: Digits indicate number of animals exhibiting reaction.

Table MA 5.2.2.2/03-5: Pharmacological and /or toxicological signs in acute inhalation toxicity study of *Beauveria bassiana* in albino rats up to 14 days after exposure at a concentration of 2.59 mg/L.

Reaction and severity	Days after exposure													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Males														
Piloerection (s-e)	5	5	1	0	0	0	0	0	0	0	0	0	0	0
Activity decrease (s-e)	5	5	2	0	0	0	0	0	0	0	0	0	0	0
Respiratory gurgle	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Death	0	0	2	2	0	0	0	0	0	0	0	0	0	0
Females														
Piloerection (s-e)	5	5	1	0	0	0	0	0	0	0	0	0	0	0
Activity decrease (s-e)	5	5	1	0	0	0	0	0	0	0	0	0	0	0
Respiratory gurgle	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Death	0	0	2	1	0	0	0	0	0	0	0	0	0	0

v – very slight

s – slight

m – moderate

e – extreme

Note: Digits indicate number of animals exhibiting reaction. If discovery was between scheduled observations, death is presented under next observation time. Time of death indicates time of discovery after death. If discovery was between scheduled observations, death is presented under next observation time.

Necropsy

Abnormal necropsy findings that occurred in animals that died on test pertained to crusted muzzle, strained/matted genital fur, discoloured lungs/liver/kidneys and empty gastrointestinal tract. The gross necropsy on the seven animals surviving to termination of the study revealed no observable abnormalities except discoloured lungs in one male and two females.

Conclusions

Beauveria bassiana strain PPRI 5339 was evaluated for its acute inhalation toxicity potential in albino rats. The acute inhalation is < 2.59 mg/L in males and females.

MA 5.2.2.2/04

Report: [REDACTED] (2011) Pilot inhalation toxicity study in rats. [REDACTED]
[REDACTED]
Unpublished Report No. CHP 076Y (Stillmeadow 15687-11)

Guidelines: None

GLP: No

Deviations: None

Aim of the Study

The aim of this study was to conduct histopathological evaluation of the lungs from both animals used in [REDACTED], 2012a (see M-MA 5.2.2.2/01).

Method

The test substance was administered to 2 male rats at 5 mg/L for 4 hours.

The lungs from both animals were submitted to Colorado Histo-Prep for histopathological evaluation by a board-certified veterinary pathologist.

The samples were trimmed, processed, sectioned and stained. Two trims from the largest lobes were submitted and one trim of each of the other 4 lobes was submitted. Histopathology of the tissues was conducted on slides stained with hematoxylin and eosin.

Results

There were moderate to severe lung lesions in both rats. In Rat No. 31M there was necrosis of the lining epithelium of secondary bronchi, terminal bronchioles and alveolar ducts. There was acute inflammation in the walls of these airways that often extended into adjacent alveoli. Many of these adjacent alveoli contained free inflammatory cells and cellular debris. There was diffuse congestion of blood vessels and alveolar walls, and many free blood cells (hemorrhage) in alveoli. The lesions were present throughout the entire lobe with essentially the same severity in all lobes submitted.

In Rat No. 32M, the lung lesions were similar to those of Rat No. 31M except not as extensive. There was necrosis of the lining epithelium in secondary bronchi, terminal bronchioles and alveolar duct with acute inflammation in the walls of these airways. The inflammation did not extend into adjacent airways as was observed in Rat No. 31M and adjacent alveoli did not have as many inflammatory cells or cellular debris. A few large bacteria were observed in the airways suggesting the rat may have had a considerable interval between death and necropsy or potentially died on study. This would account for very similar distribution of lesions, but less extensive or severe than those of Rat No. 31M. Diffuse congestion and hemorrhage was similar to that of Rat No. 31M.

Conclusion

An effect due to the test substance was present in the lungs of both rats in this study. The lesions consisted of necrosis of the lining epithelium of secondary bronchi, terminal bronchioles and alveolar ducts. There was acute inflammation in the walls of these airways and adjacent alveoli. Diffuse congestion and haemorrhage was present in all lobes. The same lesions were present in Rat NO. 32M, but were less extensive and appeared as they were perhaps of shorter duration. A few bacteria were present in the tissues suggesting this rat may have died on study or had a longer period between death and necropsy. There was essentially no difference in the lesions among the different lobes in respective rats.

MA 5.2.2.3 Intraperitoneal/subcutaneous single dose

A summary of acute intraperitoneal/subcutaneous single dose studies submitted by the registrant is presented in the following table.

Table MA 5.2.2.3-1: Summary of Intraperitoneal/Subcutaneous Single Dose Studies for *Beauveria bassiana* strain PPRI 5539

M-MA Point	Study/Route /Method	Species	Dose per Animal	Results	Conclusion	Reference
5.2.2.3/01	Acute Intraperitoneal Toxicity/Pathogenicity	Rat	7.8 x CFU/rat	No mortality or abnormal observations	Non-toxic and non-pathogenic to rat	(2012c)

In an acute intraperitoneal study conducted on *B. bassiana* strain no evidence of toxicity, pathogenicity or infectivity was observed at a dose of 1 x CFU/rat, with rapid clearance from organisms observed by day 3 post dosing. Similarly no evidence of toxicity or pathogenicity was observed following a dose of 2 x CFU/rat of *B. bassiana* strain ATCC .

It was concluded that *B. bassiana* strain PPRI 5339 is not toxic or pathogenic via the intraperitoneal/subcutaneous route of exposure.

MA 5.2.2.3/01

Report: (2012c) *Beauveria bassiana* PPRI 5339 Spore Concentrate. Acute intraperitoneal injection toxicity/pathogenicity study in rats.

Unpublished Report No. 16448-12

Guidelines: US EPA OSCPP 885.3200

FIFRA 7 USC 136

GLP: Yes

Deviations: Characterization and stability information was not provided to the testing facility.

The relative humidity was outside protocol range.

Air change is outside protocol range.

Body weights for 23 males and 2 females were outside the protocol range.

The deviations listed did not adversely affect the outcome of the study.

Aim of the study

This study was conducted to evaluate the intraperitoneal (IP) toxicity, infectivity and pathogenicity *Beauveria bassiana* strain PPRI 5339 spore concentrate a single high dose exposure and an adequate post-exposure period.

Materials and methods

Day of Treatment: 18 August and 14 September 2011

End of Experiment: 1 September and 28 September 2011

Test Item

Name: *Beauveria bassiana* strain PPRI 5339

Batch No.: S627 C2

Physical State: Light cream powder

Storage conditions: Refrigerated

Albino Sprague-Dawley rats (provided by Texas Animal Specialities, Humble, TX) in acceptable health condition were used for the study. Fifty rats (25 male and 25 female) were selected for this study.

Three groups of animals were used for this study:

Group I: Untreated control (5 animals per sex)

Group II: Inactive test substance (15 animals per sex)

Group III: Active test substance (5 animals per sex)

A 1:100 dilution of the test substance was made in a 3 step process. An aliquot of this test substance solution was autoclaved for 60 minutes at 121 °C in order to make the test substance inactivated for Group II. Following dosing to Group III, a sample of the dosing solution was used to enumerate the organisms on agar plates for dose verification.

After sterilising the injection site with alcohol, the animals were dosed with active test item (Day 0) by IP injection at a level of 7.8 x CFU/rat (15 animals per sex) or inactive (autoclaved) test substance (5 animals per sex). A treated control group was conducted concurrently.

Body weights were recorded on Day 0 (prior to dosing and on Days 7, 14 and 21).

Observations for mortality and signs of pharmacological and/or toxicological effects were conducted three times on Day 0 and after dosing and daily through to Day 21. Observations included but were not limited to; skin and fur, eyes and mucus membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behaviour patterns.

Organ weights were taken from all animals sacrificed or dying on test day except any on Day 0.

Six animals (3 per sex) from Group III were sacrificed one hour after dosing, and the peritoneal cavity from each animal was washed with 3 mL of sterile saline. The wash was recovered and plated for dosing verification and enumeration. All surviving animals were sacrificed on Day 21. A gross necropsy was conducted on each sacrificed animal at the interim times and at the termination of the study or at the time of discover after death. The gross necropsy included the following: gross observations of external surfaces, all orifices, thoracic, abdominal and pelvic cavities. Organ weights (mesenteric lymph nodes, lungs, liver, kidneys, spleen and brain) were recorded for all animals sacrificed or found dead after Day 0.

A one-way parametric analysis of variance (ANOVA) with Tukey's Multiple Comparisons Test was performed on the recorded body weights, body weight gains and the relative organ weights as a percentage of body weights. An evaluation was made of the relationship, if any, between exposure to the test substance and the incidence of severity of all abnormalities including: behaviour, body weight changes, mortality, gross lesions and toxicity, organ weight disparities, infectivity and pathogenicity.

Results

Mortality

There was no mortality in any group during the study.

Body Weights

There were no significant differences between body weights or body weight gain apart from Day 0 where male body weights for Group II were significantly greater than Group I and Group III ($p=0.0070$). This was not considered to be an effect of the test item.

Observations

There were no abnormalities observed during the course of the study.

Peritoneal Wash

For the six animals sacrificed one hour after dosing, the peritoneal cavity wash samples yielded an average colony forming unit (CFU) count of 3.0×10^4 .

Necropsy findings

The gross necropsy revealed no observable abnormalities in any group.

Organ Weights

Absolute and relative mean organ weights are presented in the following tables

Table MA 5.2.2.3/01-1: Absolute mean organ weights (g)

Group	Sex	MLN	Lungs	Liver	Kidney	Spleen	Brain
I Untreated	Males	0.1661	2.4189	18.1134	3.6460	0.9002	1.3591
II Inactive	Males	0.1783	2.3515	17.4857	3.6158	0.9846	1.4100
III Test Item	Males	0.1161	2.5636	16.8617	3.4776	1.2043	1.6191
I Untreated	Females	0.0789	1.7641	10.2321	2.1634	0.7300	1.2488
II Inactive	Females	0.1078	1.8704	10.7953	2.2160	0.7113	1.0257
III Test Item	Females	0.1036	2.1092	10.1722	2.3342	0.8731	1.2389

MLN – Mesenteric Lymph Nodes

Table MA 5.2.2.3/01-2: Relative mean organ weights (g)

Group	Sex	MLN	Lungs	Liver	Kidney	Spleen	Brain
I Untreated	Males	0.0495 %	0.7213 %	5.3998 %	1.0870 %	0.2684 %	0.4059 %
II Inactive	Males	0.0535 %	0.7034 %	5.2279 %	1.0821 %	0.2959 %	0.4219 %
III Test Item	Males	0.0346 %	0.7650 %	5.0304 %	1.0377 %	0.3609 %	0.4841 %
I Untreated	Females	0.0365 %	0.8238 %	4.7887 %	1.0141 %	0.3395 %	0.5792 %
II Inactive	Females	0.0513 %	0.8891 %	5.1608 %	1.0614 %	0.3369 %	0.4940 %
III Test Item	Females	0.0467 %	0.9461 %	4.5550 %	1.0454 %	0.3918 %	0.5486 %

MLN – Mesenteric Lymph Nodes

There were some significant differences in the relative organ weights. For males, Group III relative brain weights were significantly greater than Group I ($p=0.0230$). Group III relative spleen weights were significantly greater than Group I relative spleen weights ($p=0.0234$). Group II relative MLN weights were significantly greater than Group III MLN weights ($p=0.0209$). For females, Group II relative liver weights were significantly greater than Group II relative liver weights ($p=0.0027$). Combined spleen relative organ weights in Group III were significantly greater than Group I spleen relative organ weights ($p=0.0150$). There were no other significant differences in organ weights between groups.

Conclusions

There was no mortality in any group during the study. During observations for clinical signs, all animals appeared normal for the duration of the study. The gross necropsy conducted at termination of the study revealed no observable abnormalities.

The test substance *Beauveria bassiana* PPRI 5339 was determined to be non-toxic and non-pathogenic to rats when administered by IP injections at a single dose of 7.8×10^8 CFU/ml or 7.8×10^7 CFU/rat.

MA 5.2.3 Genotoxicity Testing

MA 5.2.3.1 *In Vitro* studies

A summary of the *in vitro* genotoxicity studies on *B. bassiana* strain PPRI 5339 submitted by the registrant is presented in the following table.

Table MA 5.2.3.1-1: Summary of in vitro genotoxicity studies for *Beauveria bassiana* strain PPRI 5539

M-MA Point	Study/Route/ Method	Test System	Results	Conclusion	Reference
5.2.3.1/01	Bacterial Reverse Mutation (AMES) OECD 471	<i>Salmonella typhimurium</i> strains TA98, TA100, TA1535, TA1537, TA102	No evidence of mutagenicity, with or without metabolic activation	Negative	Bowen, R. (2014)

In a similar bacterial reverse mutation study conducted on *B. bassiana* strain , and strain ATCC 74040, *B. bassiana* did not induce gene mutations by base pair changes or frameshifts in the genome of the bacterial tester strains and, thus, was considered to be non mutagenic.

Genotoxicity of beauvericin

Beauvericin, the most important metabolite known to be produced by strains of *B. bassiana* is a toxic cyclic hexadepsipeptide comprising a cyclic repeating sequence of three molecules of N-methyl phenylalanine alternating with three molecules of 2-hydroxyisovaleric acid. The EFSA Opinion Document on beauvericin and enniatins in food and presents a summary of available data in 2014 and summaries of the data available and general conclusions are used here to conclude on beauvericin for this submission. Whilst no beauvericin was detected in the active substance, the limit of detection (LOD) for analysis of the active substance was 0.1 mg/kg beauvericin (see MA 1.4.3), so an assessment for human health will be made assuming beauvericin content equal to the LOD.

The genotoxic potential of beauvericin was investigated in vitro in several short-term tests. Metabolic activation with S9 was not used except in the bacterial mutagenicity assays.

A summary of the results and conclusions drawn from the in vitro genotoxicity tests on beauvericin is presented in the following table.

Table MA 5.2.3.1-2: Summary of in vitro genotoxicity studies for beauvericin

Test	Cell System	Dose	Metabolic Activation	Exposure Time (h)	Result	Reference
Reverse Gene Mutation Assay (AMES)	<i>S. typhimurium</i> T A97, T A98, T A100, T A102, T A1535	0.2, 2, 20 and 500 µg/plate	With and without S9	N/A	Negative	Fotso and Smith (2003) (Doc K-MA 5.2.3.1/02)
Chromosomal aberrations	Human lymphocytes	1.25, 2.5, 5 and 10 µM	Without S9	48	Positive from 2.5 µM	Çelik et al. (2010) (Doc K-MA 5.2.3.1/03)
Micronucleus test	Human lymphocytes	1.25, 2.5, 5 and 10 µM	Without S9	48	Positive from 5µM	Çelik et al. (2010) (Doc K-MA 5.2.3.1/03)
Micronucleus test	Porcine kidney PK15 cells	0.064, 0.64 and 6.4 µM	Without S9	24 and 48	Positive at 0.64 and 6.4 µM (24 hours) and at 6.4 µM (48 hours)	Klarić et al. (2008a) (Doc K-MA 5.2.3.1/04)
Sister chromatid exchanges	Human lymphocytes	1.25, 2.5, 5 and 10 µM	Without S9	48	Positive at all doses	Çelik et al. (2010) (Doc K-MA 5.2.3.1/03)
Comet assay (alkaline)	Porcine kidney PK15 cells	0.1 and 0.5 µM	Without S9	1 and 24	Positive at 0.5 µM (24 hours)	Klarić et al. (2010) (Doc K-MA 5.2.3.1/05)
Comet assay	Human	0.1 and 0.5	Without S9	1 and 24	Slightly	Klarić et al.

(alkaline)	leucocytes	μM			positive at 0.5 μM (24 hours)	(2010) (Doc K-MA 5.2.3.1/05)
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The bacterial reverse mutation test was carried out using 5 *S. typhimurium* strains (TA97, TA98, TA100, TA102 and TA1535). In the spot test a single beauvericin concentration of 2 $\mu\text{g}/\text{plate}$ was used. A range of concentrations of beauvericin (0.2 to 500 $\mu\text{g}/\text{plate}$), both with and without S9, was used in the plate incorporation test. Three positive controls (2-aminofluorene, sodium azide and dextan) and a negative control were included in the assays. Beauvericin was non-mutagenic to all *S. typhimurium* strains, either with or without metabolic activation (Fotso and Smith, 2003) (Doc K-MA 5.2.3.1/01).

The genotoxic potential of beauvericin was investigated *in vitro* in cultures of human lymphocytes with the chromosome aberration, sister-chromatid exchange and micronucleus assays. Cytotoxicity was measured by mitotic, proliferative and nuclear division indices. A significant concentration- dependent increase was caused by beauvericin in the number of chromosomal aberrations per cell (from 1.25 μM beauvericin), sister chromatid exchanges (from 1.25 μM), and micronuclei (from 5 μM). A significant decrease the mitotic index was observed at the two highest concentrations studied (5 and 10 μM). A decrease in the proliferative and replication indices was also observed but was not significant. The authors concluded that the results indicated that beauvericin is genotoxic to human lymphocytes *in vitro* (Çelik et al., 2010) (Doc K-MA 5.2.3.1/03). The EFSA Panel on Contaminants in the Food Chain (CONTAM) noted that the effects of cytotoxicity in all these assays, particularly at the highest concentrations of beauvericin used (5 and 10 μM), cannot be . However, although the conditions of the micronucleus assay were not according to recommended guidelines, micronuclei increased in a concentration-dependent manner in human lymphocytes following beauvericin treatment.

After 24 and 48 hour treatment with 0.064, 0.64 and 6.4 μM beauvericin, Giemsa-stained binucleated Porcine Kidney PK15 cells were scored for the presence of micronuclei, nuclear buds (NBs) and nucleoplasmatic bridges. Beauvericin induced micronuclei and nucleoplasmatic bridges in a dose- dependent manner, showing predominantly clastogenic effects. A dose-dependent increase in NBs was observed after 24-hour exposure. Prolonged treatment with 5 $\mu\text{g}/\text{mL}$ of beauvericin resulted in a decrease in NBs, which according to the authors, could be related to the extrusion of micronuclei and/or saturation of the genotoxic effect (Klarić et al., 2008a) (Doc K-MA 5.2.3.1/04). The authors suggested that the effects observed may be due to oxidative stress. The CONTAM Panel noted that this would imply a threshold mode of . The CONTAM Panel also noted that the study did not give any details on cytotoxicity. However Klarić also reported in other papers that a significant decrease in cell viability preceded an increase in LDH activity observed in porcine kidney PK15 cells after treatment with beauvericin for 48 hours at a concentration of 5 $\mu\text{g}/\text{mL}$ (6.4 μM) (Klarić et al., 2006, 2008b) (Doc K-MA 5.2.3.1/06 and Doc K-MA 5.2.3.1/07), and that the using the MTT reduction assay was $5.0 \pm 0.6 \mu\text{M}$ (Klarić et al., 2010) (Doc K-MA 5.2.3.1/05). Thus the genotoxicity results at the beauvericin concentration of 6.4 μM should be interpreted with caution.

The genotoxic potential of beauvericin was investigated in porcine kidney epithelial PK15 cells and human leukocytes using the alkaline Comet assay. Genotoxicity to PK15 cells was time- and concentration dependent. Beauvericin (0.5 μM) evoked statistically significant DNA damage in PK15 cells, considering all Comet tail parameters (tail length, tail intensity, tail

moment, and abnormal sized tails (AST)) measured after 24 hours of treatment, but no significant effects were seen after treatment with beauvericin (0.1 μM), or after one hour treatment with either 0.1 μM or 0.5 μM beauvericin. The effect of beauvericin on human leukocytes was slightly concentration dependent but showed little time dependence. Tail momentum, but not tail length and tail intensity, was significantly higher in human leukocytes treated with beauvericin (0.5 μM) than in control cells after 24 hours of exposure. After one hour exposure of human leukocytes to beauvericin (0.5 μM), the changes that were observed in tail length, tail intensity and tail moment were not statistically different from control cells. No effects on comet tail parameters were seen after treatment of human leukocytes with beauvericin (0.1 μM). DNA damage changes were less pronounced in human leukocytes than in PK15 cells (Klarić et al., 2010).

Based on the available data on beauvericin, it cannot be ruled out that the substance is genotoxic. Whilst there is no *in vivo* study available on the substance to make a sufficient conclusion on genotoxicity, the registrant proposes the value of 0.15 $\mu\text{g}/\text{person per day}$, corresponding to 0.0025 $\mu\text{g}/\text{kg bw}/\text{day}$, be used as the basis for a reference value for human health risk. This value is the threshold of toxicological concern for compounds with a structural alert for genotoxicity based on the Cramer decision tree for Class III substances. This TTC value for beauvericin was derived by applying a safety factor of 10 to the standard 1.5 $\mu\text{g}/\text{kg bw}/\text{day}$ TTC recommended for a Cramer Class III substance.

MA 5.2.3.1/01

- Report:** Bowen, R. (2014) *Beauveria bassiana* PPRI 5339: Bacterial reverse mutation assay. Covance Laboratories Ltd., Otley Road, Harrogate, North Yorkshire HG13 1PY.
Unpublished Report No. 8295690
- Guidelines:** OECD Guidelines for the Testing of Chemicals No.: 471
- GLP:** Yes
- Deviations:** In order to achieve 2300g the centrifuge used was set at 3290 instead of the 300 rpm (2300 g) stated in the protocol.

Aim of the study

The objective of this study was to evaluate the potential mutagenic activity of *Beauveria bassiana* PPRI 5339 by examining its ability to revert five histidine-requiring strains of *Salmonella typhimurium* in the absence and presence of a rat liver metabolising system.

Materials and methods

Start of Experiment: 24 January 2014

End of Experiment: 17 February 2014

Test Items

Name: *Beauveria bassiana* strain PPRI 5339
Batch No.: S826

Physical State: Fine off-white powder
 Storage conditions: Refrigerated
 Purity: 1 x cfu/g (nominal)
 Expiry Date: 08 August 2014

The bacterial strains listed in Table 5.2.-13.1-33 were used in this study:

Table MA 5.2.3.1/01-1: Bacterial strains used in the evaluation of the potential mutagenic activity of *Beauveria bassiana* PPRI 5339

Organism	Strain	Type of Mutation	Mutant Gene
<i>S. typhimurium</i>	TA98	frame-shift	histidine
<i>S. typhimurium</i>	TA100	base-pair substitution	histidine
<i>S. typhimurium</i>	TA1535	base-pair substitution	histidine
<i>S. typhimurium</i>	TA1537	frame-shift	histidine
<i>S. typhimurium</i>	TA102	base-pair substitution	histidine

Test article stock formulations were formulated as a suspension under subdued lighting in DMSO. This was ultrasonicated for 5 minutes and allowed to cool prior to centrifugation at 3290 (2300g) for 5 minutes. The supernatant was membrane filter-sterilised and subsequent dilutions made with DMSO. All test article formulations were protected from light and used with approximately 4 hours of initial formulation. The concentrations listed in Table 5.2-14 were tested. 0.1 mL volume additions of test article solutions were used for all plate-incorporation treatments, 0.05 mL volume additions were used for all pre-incubation treatments.

Table MA 5.2.3.1/01-2: Concentrations of *Beauveria bassiana* PPRI 5339 tested for mutagenic activity

Experiment	S-9	Concentration of Treatment Formulation (mg/ml)	Nominal Final Concentration (µg/plate)
Range-Finder and Experiment I	- and +	0.05	5
		0.16	16
		0.5	50
		1.6	160
		5	500
		16	1600
Experiment 2	- and +	50	5000
		1.6	160
		3	300
		6.25	625
		12.5	1250
		25	2500
		50	5000

Concentration of treatment formulations used for the Experiment 2 pre-incubation treatments were twice that stated above, in order to permit treatments at the final concentrations stated, whilst volume additions were reduced to 0.05 mL.

Control treatments were performed using the same addition volumes per plate as the test article treatments. Vehicle controls comprised treatments in quintuplicate with the vehicle DMSO, and positive controls were included in triplicate without and with S-9. The positive control chemicals were used according to Table 5.2.3.1/01-3.

Table MA 5.2.3.1/01-3: Positive controls used to determine the mutagenicity of *Beauveria bassiana* PPRI 5339

Chemical	Stock Concentration (µg/mL)	Final Concentration (µg/plate)	Strain(s)	S-9
2-nitrofluorene (2NF)	50	5	TA98	-
Sodium azide ()	20	2	TA100, TA1535	-
9-aminocridine (AAC)	500	50	TA1537	-
Mitomycin C (MMC)	2	0.2	TA102	-
Benzo[a]pyrene (B[a]P)	100	10	TA98	+
2-aminoanthracene (AAN)	50	5	TA100, TA1535, TA1537	+
	200	20	TA102	+

Concentration of treatment formulations used for the Experiment 2 pre-incubation treatments were twice that stated above, in order to permit treatments at the final concentrations stated, whilst volume additions were reduced to 0.05 mL.

As the results of Experiment 1 were negative, treatments in the presence of S-9 in Experiment 2 included a pre-incubation step. Quantities of test article or control solution (reduced to 0.05 mL) were mixed together and incubated for 20 minutes at $37 \pm 1^\circ\text{C}$ protected from light for 3 days. Following incubation, these plates were examined for evidence of toxicity to the background lawn, and where possible, revertant colonies were counted.

Colonies were counted electronically using a Sorcerer Colony Counter (Perceptive Instruments) or manually where confounding factors such as bubbles or splits in the agar affected the accuracy of the automated counter.

Individual plate counts were recorded separately and the mean and standard deviation of the plate counts for each treatment were determined. Data were considered acceptable if the vehicle control counts fell within the laboratory's historical control ranges.

The presence, or otherwise, of a concentration response was checked by non-statistical analysis, up to limiting levels (e.g. toxicity or 500 µg/plate).

Results

An initial Range-Finder Experiment was used for toxicity assessment only. The background bacterial lawn was considered to be normal with no indications of a feeding effect.

Experiments 1 and 2 demonstrated no evidence of toxicity. Following *Beauveria bassiana* PPRI 5339 treatments of all the test strains in the absence and presence of S-9, no increases in revertant numbers were observed ≥ 1.5 -fold (in strain TA102), ≥ 2 -fold (in strains TA98 and TA100) or ≥ 3 -fold (in strains TA1535 and TA1537) the concurrent vehicle control. This study was considered therefore to have provided no evidence of any *Beauveria bassiana* PPRI 5339 mutagenic activity in this assay system.

Conclusions

It was concluded that *Beauveria bassiana* PPRI 5339 did not induce mutation in five histidine-requiring strains (TA98, TA100, TA1535, TA1537 and TA102) of *Salmonella typhimurium* when tested under the conditions of this study. These conditions included treatments at concentrations up to 5000 µg/plate (the maximum recommended concentration

according to current regulatory guidelines), in the absence and presence of a rat liver metabolic activation system (S-9).

MA 5.2.4 Cell Culture Study

Not required for this submission.

MA 5.2.5 Information on Short-Term Toxicity and Pathogenicity

Not required for this submission. No evidence of intrinsic toxicity or pathogenicity was observed in acute exposure studies, no further testing of toxicity is required.

MA 5.2.5.1 Health effects after repeated inhalatory exposure

Not required for this submission.

MA 5.2.6 Proposed treatment: first aid measures, medical treatment

There are no specific first aid measures for *B. bassiana*. According to the available data concerning infectivity, pathogenicity and toxicity and taking into account the rather low exposure potential, adverse health effects other than sensitisation (primarily by inhalation) upon occasional contact are unlikely. Nonetheless, standard hygienic practices and precautions should be maintained.

Inhalation, swallowing and eye contact should be avoided. Vomiting after swallowing may be dangerous due to potential aspiration.

In humans, eye, lung and disseminated infection with *B. bassiana* have been occasionally reported in the open literature. In the unlikely case of such events following contact with strain PPRI 5339, specialist's expertise and treatment with anti-mycotic drugs will be needed.

MA 5.3 Specific Toxicity, Pathogenicity and Infectiveness Studies

The following table presents a summary of specific toxicity, pathogenicity and infectiveness studies submitted by the registrant.

Table MA 5.3-1: Summary of specific toxicity, pathogenicity and infectiveness studies for *Beauveria bassiana* strain PPRI 5339

M-CA Point	Study/Route /Method	Species	Dose per Animal	Results	Conclusion	Reference
5.3/01	Dermal Pathogenicity - Topical administration semi-occlusive.	Mouse	1 x conidia/mL	No adverse effects observed	Non-toxic and non-pathogenic to rats following	Acosta-Quinetero, ME (2011)

Whilst conducted on strain LF14, the data submitted indicate that *B. bassiana* is not toxic, pathogenic or infective following topical dermal exposure.

MA 5.3/01

Reference:	Acosta-Quintero, M.E., Cazorla-Perfetti, D.J., Eduarte-Pereira, G., Morales-Moreno, P. (2011) Dermal Pathogenicity of an Autochthonous Isolate of Entomopathogenic Fungus Beauveria bassiana in Mice. Revisita Científica-Facultada de Ciencias Veterinarias, (2011) 21(6): 477-483.
Guidelines:	None
GLP:	Not applicable

Summary

This study evaluated the pathogenicity of a native isolate LF14 of *Beauveria bassiana* (Fungi: Ascomycota) following dermal administration to white mice (NMRI strain), both male and female (14 animals per sex). Twenty four hours prior to testing, mice were shaved on the ventral and dorsal trunk of the animals (~10 % of the body surface). A sterile gauze was impregnated with a suspension of 1×10^8 conidia/mL, in a single dose, for 24 hours. Control animals were administered the gauze soaked in sterile distilled water.

Clinical and behavioural evaluations, as well as body weights, were conducted daily for 14 days after exposure. The clearance of fungus from the skin was estimated by direct examination and culturing, and infectivity was assessed by performing mycological and histopathological tests after necropsies at 3, 7 and 14 days post-inoculation.

No mortality or discernible pathological or behavioural changes were observed throughout the study. In both test and control mice, there was an increasing in the mean weight gained through time, with statistically significance difference between the sexes ($p < 0,00001$). Non-germinated viable conidia were observed 24 hours after treatment, suggesting there was no germination following dermal application. No anatomic or pathological changes, or germinated conidia, were detected in any of the examined organs, suggesting no evidence for fungal multiplication.

Conclusions

It can be concluded that even following dermal application of a high dose of conidia, isolate LF14 of *Beauveria bassiana* is non pathogenic in white mice (NMRI strain).

MA 5.4 *In Vivo* Studies in Somatic Cells

Not required for this submission.

MA 5.5 Genotoxicity – *In Vivo* Studies in Germ Cells

No *in vivo* toxicity studies for genotoxicity of *B. bassiana* strain PPRI 5339 have been conducted. Based on the available *in vitro* data, no triggers for genotoxicity were observed.

The toxic metabolite beauvericin is considered potentially genotoxic based on *in vitro* data, however, the approach taken for hazard assessment has been the application of a threshold of toxicological concern value based on the genotoxic potential of the substance, no *in vivo* study has therefore been conducted, see point MA 5.2.3.1.

MA 5.6 Summary of Mammalian Toxicity, Pathogenicity and Infectiveness and Overall Evaluation

Based on original studies presented on *B. bassiana* strain PPRI 5339 and supporting publications on acute effects irrespective of the *B. bassiana* strain used, it can be concluded that strain PPRI 5339, as well as the species as a whole, is not acutely toxic to mammals, nor does it display any pathogenic or infective properties that would be a concern to humans exposed as a result of using this active substance as a plant protection product. This has been proven in experiments employing different routes of exposure such as oral, inhalative (intratracheal), intraperitoneal and topical dermal application.

In contrast, adverse effects were noted following inhalatory (aerosol and intratracheal) exposure to rats. Several studies are submitted showing significant mortality of animals following aerosol exposure of *B. bassiana* for 4 hours (█████, 2012a; █████ 2012b - see MA 5.2.2.2/01, 5.2.2.2/02). Subsequent histopathological analysis of lungs from these studies (█████, 2011 – see MA 5.2.2.2/03) showed lungs contained moderate to severe lesions, including necrosis of the lining of the epithelium of secondary bronchi, terminal bronchioles and alveolar ducts. These effects were also accompanied by acute inflammation of the walls of these airways that extended to adjacent alveoli in one animal. Many of these adjacent alveoli contained free inflammatory cells and cellular debris. There was diffuse congestion of blood vessels and alveolar walls, and many free blood cells (hemorrhage) in alveoli. The submitted studies are in contrast to acute inhalation toxicity studies conducted for other strains of *B. bassiana* (ATCC &), where no mortality was observed, but local effects of inflammation/immune reaction and organ weight increase were observed in the lungs in addition to accumulation of cellular debris. These studies were conducted using an intratracheal application method, where the test item is applied directly onto the trachea. The mortality seen in studies by █████, 2012a & 2012b, is considered to be an artefact of the test method employed, i.e. aerosol exposure for a period of 4 hours. Such an exposure regime would induce a much more extreme inflammation/immune reaction than that seen in the intratracheal exposure, due to the sheer amount of the test item filling the lungs for 4 hours. Build up of cellular debris may also have contributed to the observed mortality in the 4 hour exposure. The pathological observations in the lungs support asphyxiation as a cause of death in the animals due to inflammation and necrosis as a result of an autoimmune reaction in the lung tissue. Where a similar application method to that of the cited strains ATCC and was used, █████ 2013 showed no mortality with active PPRI 5339.

The apparent immunological response seen in animals exposed via the inhalatory route is supported by the positive result seen for skin sensitisation (█████, 2007 – see MA 5.2.1/01), although given the extreme response seen following inhalation studies this route is considered to be more critical than sensitisation elicited by dermal exposure.

Microorganisms are generally thought to have the potential to provoke sensitising reactions. The primary and supporting information submitted indicate that *B. bassiana* has the potential to induce inhalatory allergenicity and allergic skin reactions in laboratory animals and humans. Data from other *Beauveria bassiana* strains (ATCC &) indicate that this effect is not strain specific and is a mechanism for the species in general and the phrase 'may invoke a sensitising reaction' must appear on the label for this active substance.

Genotoxic potential of *B. bassiana* strain PPRI 5339 was assessed using the bacterial reverse mutation assay (Bowen 2014 – see MA 5.2.3.1/01), which was negative. However, there is a

suite of *in vitro* genotoxicity studies conducted on the major metabolite beauvericin (see Point MA 5.2.3.1) indicating positive genotoxic potential. Whilst this potential has not been confirmed in an *in vivo* study, the registrant proposes the value of 0.15 µg/person per day, corresponding to 0.0025 µg/kg bw/day, be used as the basis for a reference value for human health risk. This value is the threshold of toxicological concern for compounds with a structural alert for genotoxicity based on the Cramer decision tree for class three substances. Whilst no beauvericin was detected in the active substance, the LOD was 0.1 mg/kg beauvericin, so an assessment for human health will be made assuming beauvericin content equal to the LOD.

No further toxicity studies were conducted on *B. bassiana* strain PPRI 5339 and no further studies were deemed necessary. Whilst Regulation (EC) No 283/2013 indicates that a short-term study (minimum 28 days) is generally required for this substance, the registrant concluded that this study should be waived because no evidence of pathogenicity or infectivity was observed in the comprehensive acute data-set submitted. Additionally, the inhalation route is considered the most critical in relation to the leading health effect (sensitisation) and further long-term studies would not provide any further information to aid risk assessment and facilitate appropriate risk management measures for operators and workers.

Beauveria bassiana
strain PPRI 5339

DOCUMENT M-MA, Section 6

**RESIDUES IN OR ON TREATED PRODUCTS,
FOOD AND FEED**

Version

Date	Data points containing amendments or additions and brief description	Document identifier and version number

¹ It is suggested that applicants adopt a similar approach to showing revisions and version history as outlined in SANCO/10180/2013 Chapter 4 How to revise an Assessment Report

Table of Contents

MA 6	RESIDUES IN OR ON TREATED PRODUCTS, FOOD AND FEED	4
MA 6.1	Persistence and Likelihood of Multiplication in or on Crops, Feedingstuffs or Foodstuffs.....	4
MA 6.2	Further Information required	4
MA 6.2.1	Non-viable residues	4
MA 6.2.2	Viable residues.....	6
MA 6.3	Summary and Evaluation of Residue Behaviour Resulting From Data Submitted Under Points 6.1 and 6.2	7

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

MA 6.1 Persistence and Likelihood of Multiplication in or on Crops, Feedingstuffs or Foodstuffs

Mode of action of *Beauveria bassiana* involves destruction of its host by enzymatic action. Adherence to epicuticle surfaces of insects stimulates the infectious conidiospores to germinate. Infection commences by penetration of the germ tube producing a series of enzymes which degrade macro-molecular structures of the cuticle (chitin, lipids, proteins). Inside the host the products of further lysis processes are used by the entomopathogen for biosynthesis of fungal structures (blastospores, mycelium).

This infection mechanisms of *B. bassiana* is highly specific and strictly restricted to certain insects. Since the fungus is not capable to replicate outside its host any multiplication in or on food or feedingstuff is not expected to occur.

Beauveria bassiana does not persist in or on crops. Growth of *B. bassiana* slows down at temperatures higher than 33 °C and discontinues at > 36 °C (Fargues *et al.*, 1997) (Doc M-MA 6.1/01). It has also been shown that growth of the MPCA does not occur at temperatures of >36 °C (Neethling and Brown, 2012) (Doc M-MA 6.1/02). In addition the conidiospores of *B. bassiana* are sensitive to sunlight radiation. To this end concentrations on the surface of leaves exposed to sunlight were found to decline swiftly (Neethling *et al.*, 2014) (Doc M-MA 6.1/03).

In the absence of favourable environmental conditions and a viable host *B. bassiana* can neither thrive nor replicate. Hence the MPCA is unlikely to persist in or on crops, food and feeding stuffs in significant concentrations, i.e. concentrations which are considerably higher than under natural conditions.

MA 6.2 Further Information required

MA 6.2.1 Non-viable residues

Beside the conidiospores of *B. bassiana*, additional residual materials remaining from the fermentation process are contained in the technical grade material. These components, insoluble starch, hyphae fragments and extracellular proteins are not considered part of the non-viable residue.

In the case of *B. bassiana* strain PPRI 5339, fungal multiplication and enzymes cause disruption of functioning of the host and destroy the internal organs. There is no evidence that any secondary metabolite is involved in pathogenicity of the infestation. In open literature, *B. bassiana* is described as being able to produce a range of different metabolites such as beauvericin, bassianolide, bassiacridin, beauveriolides, beauverolides, bassianin, tenellin, oxalic acid and oosporein. Production of these metabolites depends on the culture conditions during production of the inoculum and possibly on the strain.

The mycotoxin beauvericin is considered the most important metabolite produced by *B. bassiana* (see MA 2.7). Besides a moderate insecticidal activity beauvericin has been reported to exhibit antibiotic properties but can also induce apoptosis in murine and human cell lines. However, time course of pathogenesis and mortality is consistent with a general invasive mode of infection, i.e. decomposition of internal structures of the insect host. Some authors presume that production of endotoxins like beauvericin might serve for protection of the “prey” from competing saprophytic microorganisms rather than killing the host. Apart from beauvericin there is no information available as to if the above mentioned metabolites produced by *B. bassiana* can also be formed in the particular *B. bassiana* strain PPRI 5339. Oosporine was found in related species (*B. brongniartii*) but was absent in cultures of *B. bassiana* (Strasser *et al.*, 2000) (Doc M-MA 6.2.1-01).

No residue trials have been carried out for this crop, due to the fact that no residue definition is required for *Beauveria bassiana* strain PPRI 5339.

Therefore, in order to carry out an acute risk assessment for this product, it is vital to consider the toxicity of the potential metabolite beauvericin to the end consumer.

Analysis of the production batches of *Beauveria bassiana* PPRI 5339 concentrate has demonstrated no measureable residues of beauvericin above the level of 0.5 mg/kg (Refer to MA 1.4.3). In this case, an assumption has been made that other metabolite components may also be present, of equivalent toxicity to beauvericin, and so a factor of 10 has been used to account for the potential presence of metabolites adding up to an equivalent of 5 mg/kg beauvericin per kg *B. bassiana*.

Due to the nature of the active substance, only one application is considered in the dietary risk assessment, with no pre-harvest interval.

Typical commercial yields of glasshouse grown tomatoes, cucumbers, sweet peppers and aubergines have been used to calculate a worst case residue scenario, based on the assumption that all applied active substance reaches fruit, and is not intercepted by leaves and the crop is harvested immediately after the a single application.

A recent publication by EFSA (EFSA Journal 2014;12(8):3802) has indicated that there are insufficient data to propose an acceptable daily intake (ADI) or acute reference dose (ARfD) for beauvericin, and has used the toxic threshold of concern (TTC) approach to carry out a consumer risk assessment for beauvericin in food and feed. Using this approach for this submission leads to a chronic dietary exposure limit of 0.0025 µg/kg bw/day (based on a TTC value for compounds with a structural alert for genotoxicity (Cramer Class II).

The combination of the estimation of the presence of metabolites and the Cramer Class II TTC value will give rise to a worst case estimation of the dietary risk to the consumer from beauvericin present within the formulation as applied.

The calculated concentrations of residues of beauvericin in the proposed crops arising from the use proposed use of BAS 480 00 1 is shown in Table 6.2-1.

Table 6.2-1: Calculated residues of beauvericin arising from the use of BAS 480 00 1

Crop	<i>Beauveria bassiana</i> application rate (kg/ha)	Beauvericin concentration (mg/ha)	Typical commercial yield (kg/)	Final residue of Beauvericin (µg/kg crop)
Cherry Tomatoes	0.1	0.5	26	0.0019
Round Tomatoes	0.1	0.5	65	0.0008
Cucumbers	0.1	0.5	60	0.0017
Sweet Peppers	0.1	0.5	30	0.0008
Aubergines	0.1	0.5	40	0.0013

Using this approach, it can be seen that consumption of crop treated with BAS 480 00 1 at a rate of 0.1 kg/ha is unlikely to cause any ill health effects to consumers.

MA 6.2.2 Viable residues

Beauveria bassiana is a ubiquitous entomogenous fungus which can be isolated from infected insects, soils or plants in a wide range of habitats. It may be considered as indigenous to temperate and tropical zones. The infectious mechanisms of *B. bassiana* are highly specific to insects, thus the fungus is not regarded as a plant or vertebrate pathogen.

The conidiospores of *B. bassiana* survive naturally in sheltered habitats and require specific environmental conditions of moderate temperature, high humidity and high insect population density for epizootic spread and dispersal. The natural background level of *B. bassiana* was estimated (Sheepmaker and Butt 2010, see MA 7.1.1) by calculating the 95th percentile of the geometric mean measured CFU/g soil reported in 6 studies. The resulting value of 830 CFU/g soil was considered a reasonable representation of the upper natural background levels of *B. bassiana*, as by using the 95th percentile, very high peaks were excluded. *Beauveria bassiana* was found to be susceptible to elevated temperatures (Fargues *et al.*, 1997) (Doc M-MA 6.2.2/01) and UV-light (Krieg *et al.*, 1981) (Doc M-MA 6.2.2/02). Optimal growth temperatures of the MPCA range between 25 °C and 28 °C (Neething and Brown, 2012) (Doc M-MA 6.2.2/03). At temperatures above 36 °C the conidiospores are not able to germinate or grow, with inhibition of growth commencing at temperatures higher than 33 °C. To this end *B. bassiana* is not expected to grow and multiply at mammalian or avian body temperatures.

In addition water availability is a critical parameter determining infection. At least 95 % relative humidity at the insect surface is necessary for spore germination, germ tube extension and infection to occur (Hallsworth *et al.*, 1999) (Doc M-MA 6.2.2/04). Under natural conditions of aqueous environments, the conidiospores of *B. bassiana* germinating in the absence of a suitable host insect, die within a short time (see MA 7.1.2/01). Greenhouse studies indicate that *B. bassiana* is effective for about 3 days. The study by Neethling *et al.*, 2014 (refer to MA 7.1.3/01) provides evidence that *B. bassiana* strain PPRI 5339 is highly susceptible to damage from UV light. Similarly, Edgington *et al.*, 2000 (Doc M-MA 6.2.2/05) showed that viable *B. bassiana* conidia were almost completely inactivated by exposure to 60 minutes of direct sunlight or 20 seconds of UV light at a wavelength of 302 nm.

Due to its fast degradation on plant surfaces and in soils, it is unlikely that viable residues of *B. bassiana* will occur in concentrations considerably higher than under natural conditions on the harvested food and feed.

Further studies on viable residues of *B. bassiana* strain PPRI 5339 are not considered necessary.

MA 6.3 Summary and Evaluation of Residue Behaviour Resulting From Data Submitted Under Points 6.1 and 6.2

Beauveria bassiana is an ubiquitously occurring fungus in natural soils. Its infection mechanism is strictly restricted to insects. Consequently the fungus is not regarded to persist in the environment. Infection involves destruction of the insect host by enzymatic lysis of cellular structures. Due to this specific mode of action the fungus is not regarded as a plant or vertebrate pathogen.

The conidia of *B. bassiana* are particularly sensitive to sunlight (UV radiation) and higher temperatures. Growth of the MPCA is inhibited at temperatures exceeding 33 °C and growth discontinues at more than 36 °C. For that reason growth in living mammalian and avian tissues is untenable. Any residues on crops are short-lived due to susceptibility to unfavourable environmental conditions.

As the occurrence of significant residues in or on crops, feedingstuff or food is deemed unlikely, no MRL or import tolerances have been set for *B. bassiana* strain PPRI 5339, and no residue studies have been conducted.

Strains of *B. bassiana* have the potential to produce metabolites such as beauvericin, but the amount of beauvericin or other metabolites which might be expected from the intended use is not likely to pose an acute or chronic risk for consumers.

Taking all available information into consideration, it can be concluded that the use of *B. bassiana* strain PPRI 5339 is unlikely to exert direct adverse effects on vertebrate or plant health and it is unlikely to occur in or on crops, feedingstuffs or foodstuffs in concentrations higher than those present under natural conditions. Metabolites of *B. bassiana* such as beauvericin may occur in the technical material and thus may be applied to plants. Due to the low residues in treated commodities no risk to consumers is expected from the use of *Beauveria bassiana* PPRI 5339.

Beauveria bassiana
strain PPRI 5339

DOCUMENT M-MA, Section 7

**FATE AND BEHAVIOUR IN THE
ENVIRONMENT**

Version

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Table of Contents

MA 7	FATE AND BEHAVIOUR IN THE ENVIRONMENT	4
MA 7.1	Persistence and multiplication	7
MA 7.1.1	Soil	7
MA 7.1.2	Water	9
MA 7.1.3	Air	14
MA 7.2	Mobility	18

MA 7 FATE AND BEHAVIOUR IN THE ENVIRONMENT

Introduction

PPRI 5339 is a naturally occurring, non-modified strain of *Beauveria bassiana*, originally isolated from the larva of a tortoise beetle, *Conchyloctenia punctata* (Coleoptera: Cassidinae) collected in South Africa. Further information on origin can be found in MA 2.1.2.

At the time of writing, two DARs have been produced for *B. bassiana*, strains ATCC74040 and GHA. Following ribosomal sequencing, strain PPRI 5339 was found to be 98-99% similar to the above strains, see MA 1.3.1; therefore strain PPRI 5339 can be considered essentially identical to strains ATCC74040 and GHA. As such, conclusions reported in the DARs for strains ATCC74040 and GHA are equally applicable to strain PPRI 5339 and are reproduced where necessary here.

The body of information in the open literature regarding the fate and behaviour of *B. bassiana* in the environment is extensive. The summation of these data are provided in detail in the review by Zimmermann, 2007 (Doc M-MA 7/01) and Scheepmaker and Butt, 2010 (Doc M-MA 7.1.1/01) and form the basis of the following discussions, supplemented with any other relevant literature that has subsequently been published.

The OECD 2012 guidance regarding the environmental safety evaluation of microbial biocontrol agents ENV/JM/MONO(2012)1, includes the following specific text for entomopathogenic fungi (EPF) including *B. bassiana* (emphasis underlined) and specifically recommends data in Scheepmaker and Butt, 2010 as being suitable for use to cover the soil persistence data requirements for *B. bassiana*:

“The review of Scheepmaker and Butt (2010) can be referenced in a waiver/statement to fulfill the persistence in soil data requirement for the three EPF species, B. bassiana, B. brongniartii, M. anisopliae. Since other EPF species are subjected to the same processes in the soil, it is assumed that a similar decrease of inoculum will occur in other EPF species. Therefore, the proposed methodology can be used for other mBCAs as well.

This review showed that applied inoculum of the three EPF species decreases to natural background levels in time and that increases of the inoculum are only temporary and depend on the presence of a population of host insects in the field.

A wide variety of factors explaining the decline of EPF density was described.

Some general situations with a negative impact on the survival and fate of the inoculum:

- *the microorganism is subject to competition and parasitism of the autochthon microbial community.*
- *the microorganism is subject to predator pressure.*
- *the microorganism does not germinate and/or proliferate/or multiply in the soil due to very specific (micro-) conditions.*
- *it cannot readily gain energy from hardly degradable substances of limited*

biodegradability like lignin.

It is not feasible to collect a set of background studies that are similar regarding soil condition, strain, country, crop, etc., for the simple reason that the data in the literature are not uniform and may be very limited. Moreover, in most cases, studies from the literature are not based on the desired strain for authorization, as these strains often originate from a specific isolate and can therefore only be found in a certain area. For these reasons, it is not feasible to develop standardised methods specifying the minimum number of different conditions, soils, application timings and samplings. This approach is not practicable and too costly.

Although species potentially differ in toxicity at the strain level, it is recommended to evaluate persistence at the species level as it was shown by Scheepmaker and Butt (2010) since densities of individual strains often follow a very similar decline.

It should be realized that reproduction of an entomopathogenic fungus may occur in the presence of the host. If occurring, the PEC may increase during a short period of time. After this period, a steady decline of the inoculum is expected to occur.

*In contrast to the criterion of persistence for chemicals, there is no criterion for persistence of mBCAs. From this follows that the length of the period that the applied concentration is higher than the upper background concentration is to be discussed case-by-case. This is clearly the case for *B. brongniartii* and *M. anisopliae*. In general, the persistent mBCA may be present in an inactive state, probably in a patchy distribution confined to small pockets in the soil. The mBCA may be activated under very specific conditions.”*

General information relevant to the assessment

Beauveria bassiana is an indigenous fungal species, widely distributed in natural and cultivated soils (Quesada-Moraga *et al.*, 2007, Scheepmaker and Butt, 2010 and Zimmermann, 2007) (Doc M-MA 7/02, Doc M-MA 7/02, Doc M-MA 7.1.1/01).

Beauveria bassiana is an opportunistic entomopathogenic fungi. *Beauveria bassiana* attacks its insect hosts percutaneously; the conidia of *B. bassiana* adhere to the insect cuticle by means of hydrophobic interaction between the spore wall and epicuticle lipids. A hydrophobin-type protein and certain enzymes assist in the attachment process. Germination of the conidia and the subsequent successful infection depend on a number of factors, e.g. susceptibility of the host and host stage, and certain environmental factors, such as optimal temperature and humidity. Before penetration, germ tubes may form so-called appressoria and infection pegs. The penetration process is by mechanical means and by the production of several enzymes, including proteases, chitinases and lipases, which degrade the insect cuticle. Penetration is followed by invasion, which is accompanied by several host immune response activities. Further information on mode of action can be found in MA 2.2.2 and Zimmermann, 2007.

Secondary Metabolites

During the infection process, *Beauveria* spp. produces proteolytic enzymes and toxins (see

EFSA supporting publication ¹; also see MA Point 2.7 and Zimmermann, 2007) while the host insect responds with cellular and humoral defense reactions.

Beauveria bassiana does not multiply and is metabolically inactive in the absence of host insects, and as a consequence no accumulation of potentially harmful metabolites to levels that might affect the environment is expected. Metabolic inactivity continues as long as the environmental conditions remain unchanged. Furthermore, the effect of fungistasis in non-sterile soils inhibits the germination of conidia. This has been shown by (Watson & Ford 1972) (Doc M-MA 7/03). According to (Clerk 1969) (Doc M-MA 7/04), several authors have reported that conidia of *B. bassiana* are subject to fungistatic effects in natural soils.

From this, it can be concluded that secondary metabolites including fungal toxins cannot be produced as long as the germination of conidia is inhibited, e.g. in natural soils. Many of the secondary metabolites produced during the life cycle of *B. bassiana* are produced in response to host defense mechanisms (see section MA Point 2.2.2) and therefore are unlikely to be produced without the presence of a host. Furthermore secondary metabolites are mostly peptides which are considered to be readily biodegradable (see DAR for *B. bassiana* strain GHA – Annex B.8: Fate and Behaviour in the Environment, 22 November 2007). Therefore the risk of soil contamination by secondary metabolites is considered to be negligible.

Potential effect of MPCA on analytical methods for drinking water

Despite the highly restricted persistence of *B. bassiana* strain PPRI 5339 in surface waters (see MA 7.1.2 and Doc M-MA 7.12/01) it may be theoretically possible that single conidia can reach drinking water treatment plants. This, however, is not expected to be relevant for human health as the microorganism is not pathogenic and does not produce metabolites of toxicological concern as in water it is metabolically inert. In addition, there is no evidence that the presence of *B. bassiana* strain PPRI 5339 might affect drinking water monitoring systems. Drinking water quality is monitored by screening for microbial indicator species using highly selective media on which *B. bassiana* strain PPRI 5339 does not grow. This was clearly demonstrated by the outcome of 5-batch analyses of microbial contaminants in the technical material, which included these indicator bacteria.

Potential interference with the analytical systems for the control of the quality of drinking water according to Council Directive 98/83/EC for drinking water coliforms or *E. coli*, enterococci, and *Pseudomonas aeruginosa* are monitored in drinking water.

The methods used are ISO 9308-1, ISO 16266 and ISO 7899-2. In principle, drinking water monitoring requires a concentration step (usually membrane filtration) as drinking water does not contain high densities of micro-organisms. Afterwards, the concentrated samples are subjected to cultivation procedures on media which are highly selective for the above mentioned indicator species. In most cases the media contain specific substrates which are metabolized by the indicator species resulting in a particular colour reaction which is then used as a discrimination criterion. The following media/substrates are used:

¹ Mudgal, S., De Toni, A., Tostivint, C., Hokkanen, H and Chandler, D. Scientific support, literature review and data collection and analysis for risk assessment on microbial organisms used as active substance in plant protection products –Lot 1 Environmental Risk characterisation. EFSA supporting publication 2013:EN-518 pp 87-93

- *P. aeruginosa*: acetamide reaction on cetrimid agar (fluorescing colonies) and growth at 42 °C for distinction between *P. aeruginosa* and *P. fluorescense*
- *E. coli* or coliforms: chromogenic agar (tryptone bile salts agar), red or pink colour due to glucuronidase activity
- Enterococci: Slanetz-Bartley medium (triphenyltetrazolium chloride is reduced to formazan, red to maroon colorization of enterococci colonies) and/or KAA agar (kanamycin, aesculin, azide), black colorization of enterococci colonies due to esculin-hydrolase activity

Quality assurance for the specific media always includes testing of false positive signals for other indicator species whereby in most case *E. coli*, *Enterococcus* and *P. aeruginosa* are used which never interfere with the methods used. This indicates that the media are highly specific and it is very unlikely that fungal species will grow on the media and/or exhibits the required enzymatic activities for metabolism of the applied substrates used for identification of the indicator species.

The technical material of *B. bassiana* strain PPRI 5339 was screened for the presence of the above mentioned indicator bacteria using the similar microbiological methods or identification principles. Despite a high density of *B. bassiana* strain PPRI 5339 in the test material, no false positives or any other kind of interference with the applied methods has been observed. It can be therefore concluded, that *B. bassiana* strain PPRI 5339 will not interfere with the microbiological methods used for drinking water monitoring.

Potential Effects on biological methods for sewage treatment

The relevance of sewage treatment plant (STP) assessments for microbial pesticides was discussed in the 2014 Report of the OECD/KEMI/EU Workshop on microbial pesticides: Assessment and management of risks, ENV/JM/MONO(2014)2. It was concluded that this data requirement is not relevant for microbial pesticides and therefore is not discussed further here.

MA 7.1 Persistence and multiplication

MA 7.1.1 Soil

No specific study on the *B. bassiana* strain PPRI 5339 has been conducted to evaluate its persistence in soil, however extensive literature is available on this topic. Scheepmaker and Butt, 2010 (Doc M-MA 7.1.1/01) conducted a meta-analysis of the relevant literature available at the time, and provided data concerning natural levels and persistence of *B. bassiana* in soils. Please see ENV/JM/MONO(2012)1, page 27 where the data from Scheepmaker and Butt, 2010 is considered to fulfil the persistence in soil data requirement for *B. bassiana*.

Much of the existing information was summarised in the DAR of *B. bassiana* strains GHA and ATCC-74040, the conclusions of which are considered equally applicable to strain PPRI 5339 (see MA, Point 1.3.1, PPRI 5339, ATCC74040 and GHA found to be 98-99% similar following ribosomal sequencing).

In general, it is difficult to evaluate the persistence considering only soil, because germination of *B. bassiana* conidia and subsequent multiplication only occurs in the presence of a host. A low remaining residual level in soil can be sufficient for restarting of replication on occurrence of a

host, so that from its cadaver new spores can reach the soil again. It should be kept in mind that the background levels of *B. bassiana* conidia may vary widely over one field depending on the different appearance of potential host insects and also on the possibly different microclimates. Survival depends on several abiotic and biotic factors. These are specific soil properties, temperature, moisture and water, and agrochemicals, as abiotic factors and soil micro-organisms as well as soil arthropods as biotic factors (Keller & Zimmermann 1989). It might be conceivable to find comparatively high background concentration in one place of the field and almost negligible concentration in another place being only a few meters away.

MA 7.1.1/01

Report: Scheepmaker, J. and Butt, T. (2010) Natural and released inoculum levels of entomopathogenic fungal biocontrol agents in soil in relation to risk assessment and in accordance with EU regulations. *Biocontrol Science and Technology* 20: 503 – 552.

Guideline: None

GLP: Not applicable

Summary

An overview is presented of the available data on the natural background levels of some indigenous fungal M-CAs and their persistence. The natural background level of *B. bassiana* was estimated by calculating the percentile of the geometric mean measured CFU/g soil reported in 6 studies. The resulting value of 830 CFU/g soil was considered a reasonable representation of the upper natural background levels of *Beauveria bassiana*, as by using the percentile, very high peaks were excluded.

Data from 10 studies, including laboratory and field studies, concerning the persistence of various strains of *Beauveria bassiana* was evaluated and a large level of variation was observed. However the results of the evaluated studies presented an overall trend for gradual decline of *B. bassiana* after inoculation and it was concluded that the upper natural background level was reached after 0.5 – 1.5 years.

Conclusion

Beauveria bassiana exist naturally at a background level of 830 CFU/g soil. After inoculation the population of *B. bassiana* gradually declines until reaching the background level 0.5 – 1.5 years after treatment.

Overall conclusion for soil

Beauveria bassiana strain PPRI 5339 exists at a natural background of ~1,000 CFU/g. Soil amended with exogenous *B. bassiana* strain PPRI 5339 will return to CFU levels equivalent to the natural background within 0.5 – 1.5 years. *Beauveria bassiana* strain PPRI 5339 will exist in soil as dormant conidia incapable of multiplication and metabolically inert unless there is a chance encounter with a suitable insect host, which is improbable unless at the soil surface (where it would be susceptible to inactivation via exposure to UV light, see MA 7.1.3).

MA 7.1.2 Water

The survival of *B. bassiana* strain PPRI 5339 has been studied to evaluate its potential for persistence in water (Doc M-MA 7.1.2/01).

MA 7.1.2/01

Report: Whittaker, M (2014), Hydrophobicity and survival of *Beauveria bassiana* PPRI 5339 in water. APIS, Knaresborough Technology Park, Manse Lane, Knaresborough, North Yorkshire HG5 8LF. Unpublished report APIS-BASF-010.

Guidelines: None

GLP: Yes

Summary

This study was conducted to determine the hydrophobicity and survival in water of *Beauveria bassiana* PPRI 5339 technical grade active ingredient.

Material and Methods

Test Item

Reference Name:	<i>Beauveria bassiana</i> PPRI 5339
Label Identification:	S826
Date received:	08.08.2013
Quantity received:	319.04 g
Physical State:	Off white powder
Storage conditions:	Refrigerated (~ 4 °C)
Nominal purity:	1.0 × CFU/g
Stability:	Stable at room temperature or under refrigeration

Hydrophobicity test

Approximately 1.0 g of the test item was added to approximately 50 mL of deionised water and photographed. The mixture was agitated gently for approximately 30 seconds, allowed to stand for five minutes, and then photographed again. This procedure was repeated twice more, using vigorous and then very vigorous shaking, with photographs taken after each five minute resting period.

Survival in water test

The test item was handled aseptically. Approximately 1.0 gram of batch S826 was weighed out accurately (to 1.0 mg) and mixed in the weigh boat with Tween 80 to form a smooth paste. Three further weigh boats were set up in the same manner. Each weigh boat was carefully washed into a sterile flask with 98 mL of water. Two flasks were prepared using tap water, and two with tap water that had been sterilised by autoclaving. The flasks were shaken to suspend the spores and then sonicated for 3 minutes in an ultrasonic bath.

One flask of tap water and one of sterile water were placed in an incubator set to 20°C and darkness, with the other flasks being placed on a laboratory bench in full sunlight.

At time 0 and at approximately 24 hour intervals over the following nine days, the flasks were thoroughly agitated to ensure homogeneity, and 1.0 mL withdrawn and diluted in sterile phosphate buffered saline. An aliquot of 100 µL of each dilution was plated in triplicate on Rose Bengal Agar and incubated at 28°C and read after approximately 48 hours.

Findings

Hydrophobicity test

Figures 7.1.2/01-1 to 7.1.2/01-4 show the hydrophobicity of *Beauveria bassiana* strain PPRI 5339 on application to water and then at 5 minute intervals following periods of increasingly vigorous shaking. The test item was considered extremely hydrophobic, and rapidly returned to a floating layer after shaking.

Figure 7.1.2/01-1: *Beauveria bassiana* PPRI 5339 immediately after application to water



Figure 7.1.2/01-2: *Beauveria bassiana* PPRI 5339 five minutes after gentle agitation



Figure 7.1.2/01-3: *Beauveria bassiana* PPRI 5339 five minutes after vigorous agitation



Figure 7.1.2/01-4: *Beauveria bassiana* PPRI 5339 five minutes after vigorous agitation

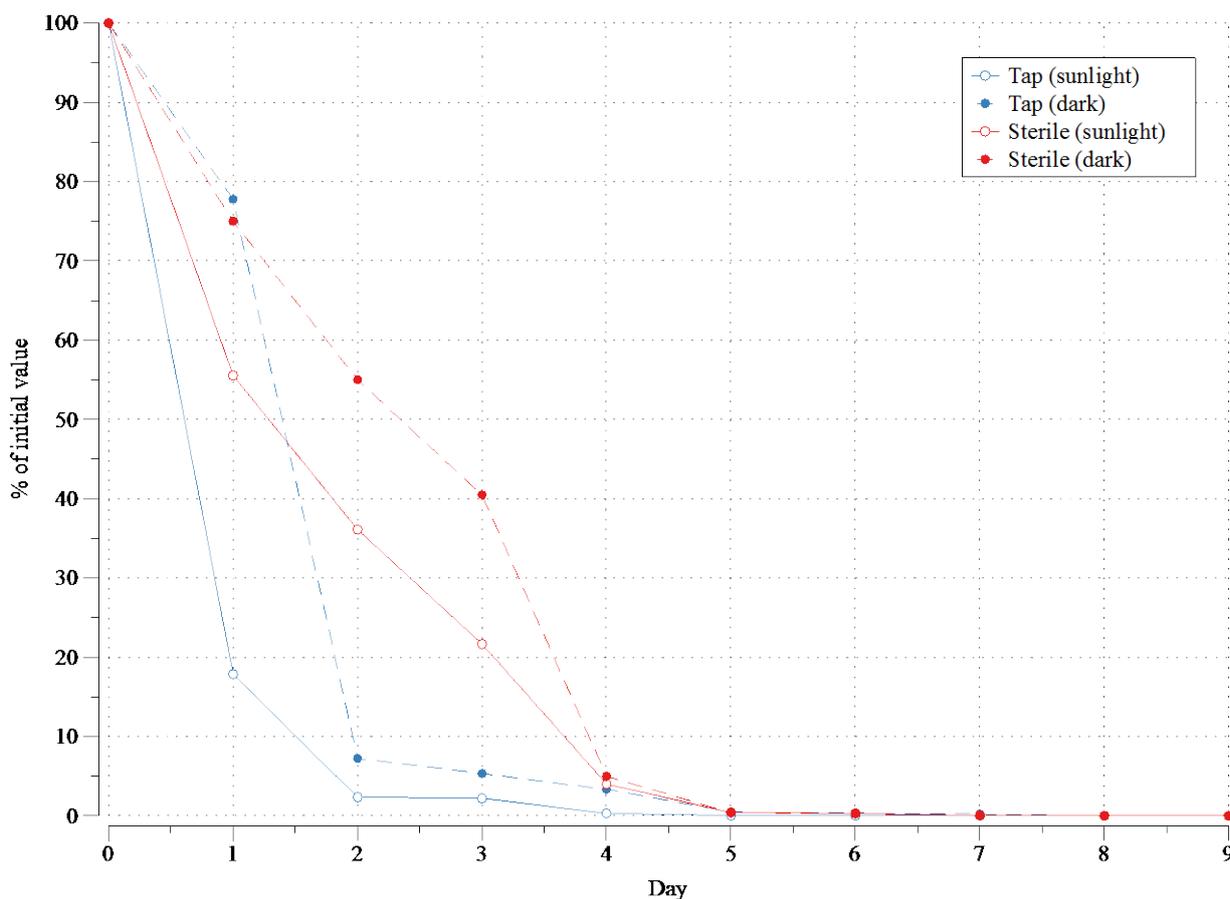
Survival in water

Table 7.1.2/01-1 and Figure 7.1.2/01-5 show the survival of the test item in water.

Table 7.1.2/01-1: *Beauveria bassiana* strain PPRI 5339 spore viability at 0-9 days after addition to water under constant dark and natural sunlight

Day	Mean viability (CFU/mL) at days after treatment				% of initial concentration			
	Tap water		Sterile Water		Tap water		Sterile Water	
	Sunlight	Darkness	Sunlight	Darkness	Sunlight	Darkness	Sunlight	Darkness
0	2.80E+11	1.80E+11	1.80E+11	2.00E+11	100	100	100	100
1	5.00E+10	1.40E+11	1.00E+11	1.50E+11	17.9	77.8	55.6	75.0
2	6.60E+09	1.30E+10	6.50E+10	1.10E+11	2.36	7.22	36.1	55.0
3	6.20E+09	9.60E+09	3.90E+10	8.10E+10	2.21	5.33	21.67	40.5
4	8.70E+08	6.00E+09	7.20E+09	1.00E+10	0.31	3.33	4.00	5.00
5	8.20E+07	9.00E+08	7.70E+08	7.60E+08	0.029	0.500	0.428	0.380
6	6.60E+07	6.70E+08	5.50E+08	6.10E+08	0.024	0.372	0.306	0.305
7	1.00E+07	4.30E+08	7.90E+07	9.50E+07	0.004	0.239	0.044	0.048
8	5.30E+06	5.40E+07	9.60E+06	8.10E+07	0.002	0.030	0.005	0.041
9	5.70E+06	8.90E+07	4.00E+06	4.80E+07	0.002	0.049	0.002	0.024

Figure 7.1.2/01-5: Reduction in percentage viable conidia of *Beauveria bassiana* strain PPRI5339 with time in aqueous solutions



The results show a slight difference in loss of viability between test solutions maintained in the light and in darkness and a more significant difference between sterile and non-sterile solutions. During the preparation of this dossier, the results were analysed to determine deactivation half-lives (, FOCUS DEGKIN v2) assuming SFO kinetics (see Q7 in Scheepmaker and Butt where the applicability of such kinetics is discussed). The values are presented in Table 7.1.2/01-2.

Table 7.1.2/01-2: Spore viability in water expressed in SFO Kinetics

Kinetic Parameter	Treatment			
	Tap water		Sterile Water	
	Sunlight	Darkness	Sunlight	Darkness
Rate constant (k)	1.729	0.715	0.580	0.437
(Days)	0.4	1.0	1.2	1.6
(Days)	1.3	3.2	4.0	5.3

Conclusions

The test item proved to be extremely hydrophobic, and rapidly formed two phases, even after vigorous mixing. By Day 9, inoculum levels had decreased to below 0.05% of the starting values in all cases, although a high number of viable spores remained.

There was evidence to suggest that the rate of reduction of viable conidia was influenced by biotic and abiotic factors; reduction was ~4 times faster in non-sterile water maintained in sunlight than in sterile water maintained in darkness.

Overall conclusion for water

Beauveria bassiana is not an aquatic fungus and is therefore not adapted to the conditions of the aqueous environment. Though survival of conidia in sterile water has been reported previously (e.g. Castellani, 1939; Boese-winkel, 1976; Müller-Kögler & Zimmermann, 1980), germination and therefore multiplication in water is not expected.

Based on the current strain specific study, it may be concluded that conidia of *B. bassiana* strain PPRI 5339 in an aquatic environment will likely remain near the surface and be subject to UV deactivation. Spores suspended in the water body will be rapidly deactivated.

MA 7.1.3 Air

The study by Neethling *et al.*, 2014 (see Doc M-MA 7.1.3/01 and summary below) provides evidence specific to *B. bassiana* strain PPRI 5339 that *B. bassiana* conidia are highly susceptible to damage from UV light.

Similarly, Edgington *et al.*, 2000 (see Doc M-MA 7.1.3/02 and summary below) showed that viable *B. bassiana* conidia were almost completely inactivated by exposure to 60 minutes of direct sunlight or 20 seconds of UV light at a wavelength of 302 nm.

Beauveria bassiana conidia are dry, small in size and released in powdery clusters and therefore are readily transported by air (Zimmermann, 2007). Fungi species use aerial spore release as the major dispersion / host location strategy and this is likely the case for *B. bassiana*. Conidia in air will be likely exposed to sunlight.

Sunlight, especially UV-B (290 - 330 nm) and UV-A (330 - 400 nm), has been demonstrated as a detrimental environmental factor affecting the field persistence of fungal insecticides (Zimmermann, 2007). The results presented in the Zimmermann (2007) review reveal that entomopathogens are inactivated within hours or days when exposed to sunlight. 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) (M-MA 7.1.3/03). After irradiation with simulated sunlight, (Ignoffo and Garcia, 1992) (Doc M-MA 7.1.3/04) 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) (Doc M-MA 7.1.3/05). In the laboratory, the survival of conidia applied in water onto glass coverslips and on crested wheatgrass was reduced by greater than 95% after 15 min exposure to UV-B radiation (Inglis *et al.* 1995) (Doc M-MA 7.1.3/06). 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* have been reported (Morley-Davies *et al.*, 1995) (Doc M-MA 7.1.3/07).

MA 7.1.3/01

Report: Neethling, D., Neethling, K. and Brown, A (2014) Effect of artificial ultra-violet light on viability of *Beauveria bassiana* strain PPRI 5339. Unpublished Report R&D061401

Guidelines: None

GLP: No

Summary

Under artificial Ultra Violet light, technical grade *B. bassiana* strain PPRI 5339 demonstrated a degradation curve typical of a *B. bassiana* and was calculated to have an t_{50} of 39 minutes.

Material and Methods

Assessments on the effect of artificial Ultra Violet (UV) light on survival of *B. bassiana* PPRI 5339 were completed as project LE326 and initiated on 03.02.2005.

Technical grade *B. bassiana* strain PPRI 5339 (Batch LE300, $1.43 \times$ viable spores/g) powder was placed in a petri dish. The petri dish was placed on the laboratory work surface 10 cm from a germicidal UV lamp producing short-wave UV radiation with a peak at 253.7 nm (UV-C). Samples were removed from the powder after 0, 10, 30, 60, 180, 240 and 285 minutes. After removal, samples were added to 10 mL of G49 wetting agent, agitated to mix and then sonicated for 3 minutes. After sonication, 0.1 mL samples were plated onto Potato Dextrose Agar plates. The plates were then incubated at 28 °C for 17 hours. Following incubation plates were counted for the number of germinated and un-germinated spores, those that had germinated had the length of the germ tube measured. The experiment was replicated twice.

Findings

Exposure of *B. bassiana* strain PPRI 5339 to UV light reduced the germination and viability of the fungal spores. After only 10 minutes the viability had reduced by 17% and at the end of the study at 285 minutes, viability had fallen to 15%. From the data, the t_{50} was estimated to be *ca* 39 minutes by linear interpolation of log transformed viable spore counts.

Table 7.1.3/01-1: Effect of exposure to artificial ultra violet under laboratory conditions on the germination and viability of spores of technical grade *Beauveria bassiana* strain PPRI 5339

Time (mins)	Average percent germination (%)	Average germ tube length (µm)
0	100.00	50.00
10	83.70	47.50
30	58.61	43.75
60	45.05	37.50
120	29.89	36.25
180	24.12	33.75
240	18.47	25.00
285	15.33	33.75

It was also shown that, of those spores that did germinate after prolonged exposure to UV light, their germ tubes did not grow as quickly over the 17 hour incubation period as those germinated spores from the untreated control that were measured after zero time exposure to UV light.

Conclusion

Rapid inactivation of *B. bassiana* spores occurred following exposure to UV light or natural sunlight.

MA 7.1.3/02

Report: Edgington, S., Segura, H., De La Rosa, W. and Williams, T (2000), Photoprotection of *Beauveria bassiana*: testing simple formulations for control of the coffee berry borer. *International Journal of Pest Management*, 2000, 46(3) 169-176

Guidelines: None

GLP: No

Summary

The effect of sunlight and UV light on the viability of *B. bassiana* conidia was assessed in a simple laboratory bioassay. Unprotected *B. bassiana* conidia were almost completely inactivated by exposure to 60 minutes of direct sunlight or 20 seconds of UV light at a wavelength of 302 nm.

Material and Methods

An isolate of *B. bassiana*, “strain 26” was used in the tests. The fungus was grown until sporulation on Sabouraud dextrose agar plus 0.5% yeast extract in sterile Petri dishes at ambient laboratory temperatures, 26 - 30°C. Spores were harvested from the plates and stored in a desiccator at 4°C. Batches of spores showing less than 80% germination in control tests at any time during testing were discarded.

Sunlight test

Spores were suspended to a concentration of approximately $1 \times$ spores/mL in 0.1% Triton X-100. A 3 μ L droplet of this suspension was smeared evenly across the length of a clean glass microscope slide. Spores were exposed to direct natural sunlight when sun intensity was at a maximum.

Following exposure, a thin layer of 3% Sabouraud agar (+0.02% aureomycin) was added onto each slide and all were then placed in a dark humid box for 18 h at ambient laboratory temperatures (26 - 30°C). Germination was determined using a phase contrast microscope at $\times 600$ magnification. The mean percent spore germination was calculated for each slide by counting the number of germinated and ungerminated spores in five fields of view. Between three and six replicate slides were used for each treatment.

UV light test

Slides holding spores were prepared as above with the exception that various chemical with potential UV protection were included. Spores were exposed to UV light emitted from a trans illuminator, peak 302 nm. Test slides were located at a distance of 2 cm from the UV filter, and exposed for between 5 and 25 seconds. Spore germination was assessed as previously described.

Findings

Sunlight test

There was a highly significant negative relationship between spore germination and duration of exposure to sunlight ($P < 0.0001$). Unprotected spores were inactivated rapidly; after 60 minutes exposure spore germination had been reduced from an initial mean of 89.4% (range of SE 90.5 - 88.3%) to just 2.8% (range of SE 3.4 - 2.3).

UV light test

Even in the presences of UV protection, spore inactivation was extremely rapid and was linearly correlated to the duration of exposure ($P < 0.0001$). After just 20 seconds of UV irradiation, mean spore germination fell from an original 95.3% (range of SE 94.9 - 95.7%) to 0.74% (range of SE 0.92 - 0.59%).

Conclusion

Rapid inactivation of *B. bassiana* spores occurred following exposure to UV light or natural sunlight.

Overall conclusion for air

In the absence of a specific host insect, viability of conidia of *B. bassiana* in the air or epigeal habitats is mainly affected by the UV portion of the solar spectrum. On the basis of the persistence data, significant degradation of *B. bassiana* conidia on leaves under field conditions may occur in less than 1 day. *Beauveria bassiana* spores released to air are not persistent and unlikely to remain viable for extended times necessary for long range transport.

MA 7.2 Mobility

In Soil:

The following study (see Doc M-MA 7.2/01) is summarised here to provide indication of where in soil conidia of *B. bassiana* can be found after a spray application, according to the DAR the study is not applicable for the analysis of leaching in soil, but it does provide data regarding the position of viable conidia in soil columns post-irrigation.

MA 7.2/01

Report: Storey, G.K. and Gardner W.A., Movement of an aqueous spray of *Beauveria bassiana* into the profile of four Georgia soils, *Environmental entomology* 17(1): 135 – 139 (1988)

Guidelines: None

GLP: No

Summary

The vertical dispersion of conidia of *B. bassiana* in soil was assessed in a greenhouse and field situation, post irrigation. Conidia were found to be not very mobile in soil and generally remain on the surface of the soil. The movement of conidia vertically, through the soil profile, is positively correlated with high infiltration rates in soil.

Material and Methods

Four soil series obtained from various locations in Georgia were used in this experiment. They were as follows: The Cecil series (sandy clay loam soil), the Tifton series (sandy loam soil), the Greenville series (sandy clay loam soil) and the Townley series (clay loam soil).

In a greenhouse study, an intact soil block (20 cm deep) from each of the four Georgian locations was removed. They were then placed in a greenhouse and maintained at 30°C, under a polyethylene tent. Each container was covered with a plastic tray to reduce moisture loss from the blocks. A 100 mL aliquot of an aqueous suspension of commercially formulated *B. bassiana*, containing 9.44×10 CFU/mL, was sprayed on the surface of each block.

A soil core from each soil series was removed prior to and 72 h after application. Horizontal sub samples of each of these cores at 0, 4, 8, 12 and 16 cm depth were washed. Dilutions of each wash were then drop-plated on oatmeal-dodine agar and incubated at 25 °C for 7 days. Vertical movement estimates were based on the number of CFUs recovered from each of dry soil at each depth of the respective soil cores.

In a field study, field plots measuring 1.8 by 1.8 metres at each of the four Georgian locations were treated with 2 L of an aqueous suspension of a commercial product, containing conidia of *B. bassiana*. This was followed by application of 8 L of water to each plot.

Soil cores (15 cm deep) were removed from each of the plots prior to and 2 h after application of the conidia. A 1 g sub sample was removed from three 5 cm vertical sections of each core and processed for CFU enumeration as previously described. Vertical movement was estimated by the number of CFU recovered from each depth of the various soil profiles examined.

The field experiment was conducted in Canada (Leathbridge) from July 28 (trial one) and August 12 (trial two) in 1993. The target concentration was 3 x water (at a rate of 100 L/ha) and 4 oil (at a rate of 5 L/ha) compatible formulations. After spraying ten leaves from the top canopy were randomly sampled at time 0, 1, 2, 4, 6, 8, 12, and 16 days. The CFU/ was determined. All relevant weather and climate parameters were measured during the experiment.

Findings

In greenhouse studies, 95% of the total number of CFUs recovered in core samples of the Townley, Greenville and Cecil series soils remained at the surfaces. In the Tifton series soil, 87 % of the total CFU were recovered from the surface with an additional 9 % recovered 4 cm below the surface. Vertical movement of conidia in these soils appears to be greatest in soils with high infiltration rates, such as the Tifton and Greenville series soils. Further details on conidial movement through the four soil series in a greenhouse study are presented in Table 7.2/01-1.

Table 7.2/01-1: Number of CFUs of *Beauveria bassiana* per cubic centimeter dry soil recovered from selected depths 72 h after application to soil blocks

Depth (cm)	Soil			
	Cecil	Greenville	Townley	Tifton
0	1.13E+08	3.29E+07	2.24E+07	1.44E+07
4	1.20E+05	1.41E+05	3.80E+04	1.50E+06
8	2.80E+04	1.90E+04	7.00E+03	2.70E+05
12	3.70E+04	1.70E+04	4.10E+04	2.86E+05
16	1.07E+05	5.00E+04	1.70E+04	1.60E+04

In a field study, core samples taken 2 h after application indicated that the four soils restricted vertical movement of the conidia. Greater than 94% of the total number of CFU recovered in the cores ranged from the upper 5 cm of the profile of each soil series. At the 5 – 10 cm soil depth, 0.7, 1.8, 3.3 and 4.4 % of the total CFU were recovered from the Cecil, Greenville, Tifton, and Townley series soils, respectively. Retention of the conidia in the upper profile of these soils appears to be caused by mechanical filtration within the soil structure. No significantly correlation between vertical movement and the sand, silt, or clay content was found.

Further details on conidial movement through the four soil series in a field study are presented in Table 7.2/01-2

Table 7.2/01-2: Number of CFUs of *Beauveria bassiana* per cubic centimeter dry soil recovered from selected depths 2 h after application to soil blocks

Depth (cm)	Soil			
	Cecil	Greenville	Townley	Tifton
0-5	3.62E+06	4.97E+06	1.85E+06	4.65E+06
5.1 – 10.0	2.40E+04	9.30E+04	8.50E+04	1.61E+05
10.1 – 15.0	4.00E+03	9.10E+04	1.50E+04	4.50E+04

Conclusion

Conidia of *Beauveria bassiana* are not very mobile in soil and generally remain on the surface of the soil. The movement of conidia vertically, through the soil profile, is positively correlated with high infiltration rates in soil.

MA 7.2/02

Report: Kim, J.S. Skinner, M. Gouli, S. Parker, B.L., Influence of top-watering on the movement of *Beauveria bassiana*, GHA (Deuteromycota: Hyphomycetes) in potting medium, *Crop Protection* 29 (2010) 631–634

Guidelines: None

GLP: No

Material and Methods

Beauveria bassiana strain GHA was obtained from the Entomology Research Laboratory Collection at the University of Vermont. The isolate was stored in 10% (v/v) glycerol at -80 °C, and grown on quarter-strength Sabouraud dextrose agar plus yeast at 25 ± 1 °C in darkness for 14 days.

A millet grain based solid culture method was used to produce mycotized *B. bassiana* GHA grains, with a concentration of $1.1 \times$ conidia per gram and a germination rate of 98.2% at 24 h and 20 ± 1 °C. The cultures were dried until the moisture content of the conidia powder was less than 5%.

Into 36 plant pots, 135 g of potting medium (sphagnum peat moss, vermiculite, bark, fine bark, starter nutrient charge, dolomitic limestone and wetting agent) was added. Three gravimetric moisture content treatments were tested, 43, 100 and 233% of water saturation, each replicated three times. Into each saturation treatment replicate, 2 g of mycotized millet grains were added and mixed into the upper 3 cm of the medium. Three pots of each treatment received no fungus and served as controls. Treatment and control pots were placed in individual saucers to collect water held at room temperature (25 ± 2 °C). To simulate standard watering practices, every second day after fungal application, pots were top-watered with either 50, 100 or 150 mL over a 18 day period. Control pots received 150 ml of water. Thirty minutes after watering, the amount of released water in each saucer was measured. The number of colony forming units (CFU) of *B. bassiana* was determined by plating the released water on a *B. bassiana* selective medium.

After 18 days the potting medium in each pot was separated into ~ 3 cm layers representing upper, middle and lower layers. From each layer a 20 g subsample was taken and mixed with a 0.02% solution of polysiloxane polyether to make 50 mL soil suspensions with even distribution of *B. bassiana*. Another 5 g subsample from the same layer was taken to measure dry weight.

Findings

No *B. bassiana* CFUs were detected in water from control pots throughout the experiment. Therefore, it is assumed that *B. bassiana* CFUs from pots treated with the mycotized grains were GHA.

For the 233% saturation with the 100 mL and 150 mL top watering treatments, a small number of *B. bassiana* CFUs were detected on days 0, 2 and 4 post application in the water released, though differences were not significant between these treatments ($P > 0.05$). A small number of *B. bassiana* CFUs were also detected from water released on days 2 and 4 post-application in the treatments with 43% and 100% saturation and the 150 mL top-watering. On day 6 post-application, a very small amount of *B. bassiana* CFUs were detected from the 43% saturation with the 100 ml top watering treatment, less than $1 \times$ CFUs per pot. No *B. bassiana* CFUs were found after day 6 for all treatments. Overall, very few *B. bassiana* CFUs ($<1 \times$ /pot) were released, when compared with the number of conidia ($2.2 \times$ conidia/pot) applied at the start of the study.

Significantly more *B. bassiana* CFUs were detected in the upper than in the middle and lower layers for all moisture content treatments tested.

In the upper layer of the 233% saturation treatment, significantly fewer CFUs were found from the 100 and 150 mL watering regime than the 50 mL regime ($P < 0.001$). In addition, when comparing the number of CFUs among percent saturation treatments, significantly fewer were detected in the 233% treatment than the others ($P < 0.001$ in both cases). When data from the number of CFUs detected in the released water after top-watering and the fungal concentration in the three layers of medium in the pots were considered together, the results suggest that by day 6–8 the fungus had inclusively colonized the upper layer of the medium (we observed white mycelium on the potting medium), thereby eliminating any leaching of conidia from water at the bottom of the pot. Minimal fungal growth was detected in the middle and lower levels of the potting medium regardless of the percent saturation or watering regime. Three factors may explain this outcome: (a) fungal colonization of soil or potting media occurs relatively slowly, and 18 d was insufficient time for the fungus to grow throughout the pot, i.e. to the middle and lower layers; (b) fungal colonization is inhibited by abiotic factors such as poor aeration or unsuitable pH conditions; (c) fungal movement is prevented by the filtering effect of the potting medium components such as vermiculite or dolomitic limestone.

Conclusion

In conclusion, a small amount of the *B. bassiana* GHA applied to the upper layer of the potting mix was detected in water at the bottom of the pots, and after 4–6 d, no evidence of fungal leaching was found. After 18 days of regular top-watering, the concentration of *B. bassiana*, based on the number of CFUs, was greatest in the top 3 cm of potting medium, though the percent saturation of the medium and the volume of water it received affected fungal

concentration levels. The potting medium with a consistent saturation of 233% is not common in greenhouse production as it encourages root rot and poor plant growth.

Overall conclusion for mobility in soil

Conidia which have a waxy non-wetting coat (as shown in MA 7.1.2/01) remain on the soil surface (Burgess 1950). Therefore conidia of *B. bassiana* are not very mobile in soil and generally remain on the surface of the soil. Furthermore, no groundwater risk assessment is necessary since *B. bassiana* is neither pathogenic nor toxic to humans.

In Air:

Many fungi species use aerial spore release as the major dispersion / host location strategy and this is likely the case for *B. bassiana*. *Beauveria bassiana* conidia are dry, small in size and released in powdery clusters and therefore are readily transported by air (Zimmermann, 2007).

Rapid inactivation of *B. bassiana* spores occurred following exposure to UV light or natural sunlight (see MA, Point 7.1.3) and therefore transport in air is considered insignificant.

In water:

Conidia are hyaline, globose to broadly ellipsoidal, generally $2 - 3 \times 2 - 2.5 \mu\text{m}$ (Zimmermann, 2007). The globose or ovoid shape of conidia is considered not suitable for efficient transport in water (see also EFSA conclusion on pesticide peer review²).

² Conclusion on the peer review of the pesticide risk assessment of the active substances *Beauveria bassiana* strains ATCC-74040 and GHA1. EFSA Journal 2013;11(1):3031

***Beauveria bassiana* strain
PPRI 5339**

DOCUMENT M-MA, Section 8

EFFECTS ON NON-TARGET ORGANISMS

Version

Date	Data points containing amendments or additions and brief description	Document identifier and version number

¹ It is suggested that applicants adopt a similar approach to showing revisions and version history as outlined in SANCO/10180/2013 Chapter 4 How to revise an Assessment Report

Table of Contents

MA 8	EFFECTS ON NON-TARGET ORGANISMS	4
MA 8.1	Effects on Birds	5
MA 8.2	Effects on Aquatic Organisms.....	7
MA 8.2.1	Effects on fish	7
MA 8.2.2	Effects on freshwater invertebrates	7
MA 8.2.3	Effects on algae growth.....	8
MA 8.2.4	Effects on plants other than algae.....	8
MA 8.3	Effects on Bees.....	9
MA 8.4	Effects on Arthropods Other Than Bees	36
MA 8.5	Effects on Earthworms.....	44
MA 8.6	Effects on Non-Target Soil Micro-Organisms	45
MA 8.7	Additional Studies	47

MA 8 EFFECTS ON NON-TARGET ORGANISMS

Introduction

PPRI 5339 is a naturally occurring, non-modified strain of *Beauveria bassiana*, originally isolated from the larva of a tortoise beetle, *Conchyloctenia punctata* (Coleoptera: Cassidinae) collected in South Africa. Further information on origin can be found in MA, Point 2.1.2.

Beauveria bassiana is an opportunistic entomopathogenic fungi. *Beauveria bassiana* attacks its insect hosts percutaneously; the conidia of *Beauveria bassiana* adhere to the insect cuticle by means of hydrophobic interaction between the spore wall and epicuticle lipids. A hydrophobin-type protein and certain enzymes assist in the attachment process. Germination of the conidia and the subsequent successful infection depend on a number of factors, e.g. susceptibility of the host and host stage, and certain environmental factors, such as optimal temperature and humidity. Before penetration, germ tubes may form so-called appressoria and infection pegs. The penetration process is by mechanical means and by the production of several enzymes, including proteases, chitinases and lipases, which degrade the insect cuticle. Penetration is followed by invasion, which is accompanied by several host immune response activities. Further information on mode of action can be found in MA, Point 2.2.2.

During the infection process, *Beauveria* spp. produces proteolytic enzymes and toxins¹ (also see MA, Point 2.7) while the host insect responds with cellular and humoral defense reactions. In the insect body, the fungus multiplies as blastospores, or yeast-like cells, which are distributed passively in the haemolymph. Enzymes begin to destroy the internal structures of the host insect causing morbidity within 36 to 72 hours. Reduced feeding and immobility are rapidly evident, and the insect dies within 4 to 10 days post-infection. The time to death will depend on the insect species, age and conidial dose. After death of the insect, the fungus starts its saprophytic growth: blastospores transform into mycelia, which emerge through the cuticle. Aerial conidia are formed on the surface of the insect cadaver, which build the characteristic white mould. Sporulation occurs only in conditions of high humidity.

Beauveria bassiana does not multiply and is metabolically inactive in the absence of host insects, and as a consequence no accumulation of potentially harmful metabolites to levels that might affect the environment is expected. Metabolic inactivity continues as long as the environmental conditions remain unchanged.

¹ Mudgal, S., De Toni, A., Tostivint, C., Hokkanen, H and Chandler, D. Scientific support, literature review and data collection and analysis for risk assessment on microbial organisms used as active substance in plant protection products –Lot 1 Environmental Risk characterisation. EFSA supporting publication 2013:EN-518 pp87-93

MA 8.1 Effects on Birds

No specific studies on *Beauveria bassiana* strain PPRI 5339 have been conducted on birds.

The table below summarises toxicity data derived for birds from various sources. A review paper by G. Zimmermann (Zimmermann, 2007 (Doc M-MA 8.1/01) covers a number of these sources.

Table 8.1-1: Summary of avian toxicity data

Species	Test type	MCPA	Endpoint	Reference/Source
Bobwhite quail (<i>Colinus virginianus</i>)	5 day dosing/25 days observation	<i>Beauveria bassiana</i> strain ATCC 74040	>2667 mg/kg bw/day corresponding to $6.85 \times$ CFU/kg bw/day (No observed external signs of pathogenicity. No findings of gross necropsy)	EFSA Journal 2013;11(1):3031
American kestrel (<i>Falco sparvensis</i>)	Dietary field test	<i>Beauveria bassiana</i> strain GHA	>2.5 \times conidia/kg body weight. No adverse effects on growth or body mass	Althouse <i>et al.</i> , (1997)
Ring-necked pheasants (<i>Phasianus colchicus</i>)	Dietary test in which birds were fed with spores and infected grasshoppers		No significantly different weight gain and no detectable histopathological changes	Johnson <i>et al.</i> , (2002)
Bobwhite quail	5-day oral toxicity	<i>Beauveria bassiana</i>	>2000 mg/kg/day	Copping (2004)
Chicken (<i>Gallus domesticus</i>)	5-day dietary toxicity	<i>Beauveria bassiana</i>	No adverse effects on mortality, behaviour or weight at conidia of <i>Beauveria bassiana</i> /g food	Haas-Costa <i>et al.</i> (2010)

LIT = Literature review conducted in 2014

Zimmermann conducted a review on the safety of *Beauveria bassiana* and *Beauveria brongariartii*, published in 2007 (Doc M-MA 8.1/01). A summary of the key points derived from this review are given below:

Young American kestrels (*Falco sparvensis*) were fed with $5 \times$ or $2.5 \times$ spores of *Beauveria bassiana*/kg body weight in field experiments. No adverse effects on growth, body mass or survival were apparent (Althouse *et al.*, 1997) (Doc M-MA 8.1/02). Male and female ring-necked pheasants (*Phasianus colchicus*) were dosed with conidia of *Beauveria bassiana* strain GHA and with *Beauveria bassiana* strain GHA infected grasshoppers. In both sexes, the weight gain at 17 and 25 days was not significantly different between treated and control groups. Histopathological changes were generally undetectable (Johnson *et al.* 2002) (Doc M-MA 8.1/03). No side effects on birds, especially young ones were detected (Havelka & Ruge 1988) (Doc M-MA 8.1/04). According to Copping (2004) (Ref M-MA 8.1/05), the non-target avian toxicity for *Beauveria bassiana* is: oral (5 days) for quail > 2000 mg/kg daily (by gavage).

Much of this information was summarised in the DAR of *Beauveria bassiana* strains GHA and ATCC-74040, the conclusions of which are considered equally applicable to strain PPRI 5339 (see MA, Point 1.3.3 PPRI 5339, ATCC74040 and GHA found to be 98-99% similar following

ribosomal sequencing).

A relevant study arising from a literature review is summarised below.

MA 8.1/06

Report: Haas-Costa, J, Alves, LFA and Daros, AA (2010). Safety of *Beauveria bassiana* (Bals.) Vuill. to *Gallus domesticus* L. *Brazilian Archives of Biology and Technology*, (2010) 53(2): 465-471

Guidelines: None

GLP: No

Summary

The effect on the feeding, behaviour, histology of the digestory system and anatomy of *Gallus domesticus* when treated orally with *Beauveria bassiana* (strain Unioeste 4) was assessed. There was 100% of survival of the birds, and no behavioural alterations or external lesions were found.

Materials and methods

The methodology used was based upon the “Protocolo de avaliação de agentes microbianos de controle de pragas para registro como bioinseticidas”.

Eleven day old male chickens were used for the study. They were acclimatised for three days at 20 to 30°C and a 16 h photoperiod.

The birds were offered either food containing the active fungus at conidia/g food, inactive fungus or untreated food. Ten birds were used per treatment. The birds were treated daily for five days. Between each feeding the birds were fasted for 15 hours.

Chicken faeces were collected, and the birds were observed for 28 days. Weight changes of the birds, as well as any sign of intoxication or pathological modification were also evaluated. Tissue samples were withdrawn in order to examine lesions (with the aid of an optical microscope).

Findings

There was 100% survival of the birds, and no behavioural alterations or external lesion were found. The group treated with the active fungus gained the most weight. Viable conidia were observed in the faeces until 24 h after feeding the fungus, indicating that there was no germination inside the digestory system. No tissue lesion was observed.

Conclusions

No mortality or adverse effects on behaviour or weight were observed for chickens (*Gallus domesticus*) fed with conidia of *Beauveria bassiana*/g food.

MA 8.2 Effects on Aquatic Organisms

Studies with aquatic organisms have not been conducted with aquatic organisms as they are not required for glasshouse applications. Also, in an aquatic environment conidia of *Beauveria bassiana* strain PPRI 5339 will be expected to inactivate rapidly in natural water bodies (see M-MA 7.1.2).

MA 8.2.1 Effects on fish

Beauveria bassiana strain PPRI 5339 is currently intended for glasshouse use only and no aqueous exposure is anticipated, as such no specific studies are necessary for a risk assessment. A fish study is underway with PPRI 5339, anticipating non-protected future uses and the reports will be available in 2015.

Zimmermann conducted a review on the safety of *Beauveria bassiana* and *Beauveria brongariartii*, published in 2007 (Doc M-MA 8.1/01). A summary of the key points derived from this review are given below:

Safety tests against fish for *Beauveria bassiana* were reported for the isolate GHA by Goettel and Jaronski (1997) (Doc M-MA 8.2.1/01) and for the product Naturalis-L (ATCC 74040) by Copping (2004) (Ref M-MA 8.1/05). No adverse effects of strain GHA were observed in embryos and larvae of the fish *Pimephales promelas*, when exposed for 31 days to $1 \times$ CFU/L. Naturalis did not affect fish embryos, larvae or adults; the (31 days) for rainbow trout was 7300 mg/L. In contrast, when developing embryos of the inland silverside fish, *Menidia beryllina*, were exposed to conidia of *Beauveria bassiana*, various adverse effects were observed in embryos and larvae (Genthner & Middaugh 1992 (Doc M-MA 8.2.1/02); Middaugh & Genthner 1994 (Doc M-MA 8.2.1/03)).

MA 8.2.2 Effects on freshwater invertebrates

Beauveria bassiana strain PPRI 5339 is currently intended for glasshouse use only and no aqueous exposure is anticipated, as such no specific studies are necessary for a risk assessment. A freshwater invertebrate study is underway with PPRI 5339, anticipating non-protected future uses and the reports will be available in 2015.

Zimmermann conducted a review on the safety of *Beauveria bassiana* and *Beauveria brongariartii*, published in 2007. A summary of the key points derived from this review are given below:

No toxicity or pathogenicity was observed for *Daphnia magna* when exposed to $1 \times$ conidia of *Beauveria bassiana* strain GHA/L for 21 days (Goettel & Jaronski 1997) (Doc 8.2.2/01). Strain GHA was also not infectious against the grass shrimp, *Palaemonetes pugio*, after percutaneous and oral contamination (Genthner *et al.* 1994b) (Doc 8.2.2/02). In the mysid shrimp *Americamysis bahia* (formerly *Mysidopsis bahia*) *Beauveria 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) (Doc 8.2.2/03).

Beauvericin has been found to be highly toxic towards *Artemia salina* larvae and murine cell

lines and can induce apoptosis (Pascale *et al.* 2002). The effects of beauvericin on mosquito (*Aedes aegypti*) larvae was investigated by Groves & Pople (1980)(Doc 8.2.2/05). At a concentration of 10 mg/L, 10%, 39% and 64% mortality was recorded after 18, 48 and 72 h, respectively. At the higher concentration of 20 mg/L, 58% and 86% mortality was recorded after 18 and 48 h, respectively. In the mysid *A. bahia*, beauvericin, Genthner *et al.* (1994a, Doc 8.2.2/03) found the to be 0.56 mg/L.

According to Zimmermann, there are no published studies regarding effects of other *Beauveria* metabolites as well as of *B. brongniartii* on aquatic organisms.

MA 8.2.3 Effects on algae growth

Beauveria bassiana strain PPRI 5339 is currently intended for glasshouse use only and no aqueous exposure is anticipated, as such no specific studies are necessary for a risk assessment. An algae growth study is underway with PPRI 5339, anticipating non-protected future uses and the reports will be available in 2015.

MA 8.2.4 Effects on plants other than algae

Data are not required as *Beauveria bassiana* strain PPRI 5339 is not a plant pathogen and there are no reported adverse effects on terrestrial plants by any strains.

MA 8.3 Effects on Bees

Table 8.3-1: Summary of bee toxicity data

Species	Test type	MCPA	Endpoints	Reference/Source
Honey bee (<i>Apis mellifera</i>)	10-day oral exposure	<i>Beauveria bassiana</i> strain PPRI 5339	>50% mortality in inactive control 3 days after treatment ^a	Younger (2012) /BASF study
	16-day oral exposure		40% mortality in inactive control 16 days after treatment ^a	Younger (2012) /BASF study
	10-day oral and contact exposure		>10 days; >MHC for oral and contact dose	Whittaker 2014/ BASF study
Bumble bee (<i>Bombus terrestris</i>)	10-day contact exposure		>10 days; >3.1 × CFU/bee	Whittaker 2013/ BASF study
	10-day oral exposure		>10 days; >2.9 × CFU/bee	Whittaker 2013/ BASF study
Honey bee	10-day contact exposure		>10 days; >2.9 × CFU/bee	Whittaker 2013/ BASF study
	10-day oral exposure	>10 days; >9.57 × CFU/bee	Whittaker 2013/ BASF study	
	Field study – whole colony exposure with test substance added directly to the hive	<i>Beauveria bassiana</i> isolate 05002	No adverse effects on colony health at treatment rates ranging from 1.72 × CFU/g to 3.70 × CFU/g.	Meikle <i>et al.</i> (2008)
	Field study – whole colony exposure with test substance added directly to the hive	<i>Beauveria bassiana</i> isolate 05002	No adverse effects on colony health at treatment rates ranging from 6.33 × CFU/g to 1.96 × CFU/g.	Meikle <i>et al.</i> (2008)/LIT
Bumble bee	Laboratory oral and contact exposure	Not specified	>3.45 × CFU/g	EFSA Journal 2013;11(1):3031
	Evaluation of the effects on health to bees used as vectors	Prestop-Mix, based on <i>G. catenulatum</i> J1446	No adverse effects on bumble bee workers	Mommaets <i>et al.</i> (2011)
Bumble bee (<i>Bombus impatiens</i>)	Oral and topical exposure	<i>Beauveria bassiana</i> (Botanigard®)	Oral: no adverse effects from 1.16 × to 4.66 × CFU/L Topical: no adverse effects from 2.33 × to 4.66 × CFU/L	Mommaets <i>et al.</i> (2011)
Bumble bee (<i>Bombus impatiens</i>)	Oral and topical exposure	<i>Beauveria bassiana</i> (Botanigard®)	Oral: no adverse effects at 2.5 × CFU/L Topical: adverse effects at 2.5 × CFU/L	Ramanaidu & Cutler, (2013)

^a This study will not be included in the risk assessment but has been summarised below. The reasons for its exclusion

LIT = Literature review conducted in 2014

Summaries of studies conducted with *Beauveria bassiana* strain PPRI 5339 are presented below:

MA 8.3/01

Report: Younger C (2012). *Beauveria bassiana* PPRI 5339 spore concentrate. Honey Bee, *Apis mellifera* non-target microbial testing. Stillmeadow Inc., 12852 Park One Drive, Sugar Land TX 77478. Study Code: 16339-12

Guidelines: OCSPP 885, 4380

GLP: Yes

Deviations: The provided Certificate of Analysis was not accompanied by a GLP compliance statement.
Stability information was not provided to the testing facility.
Mixture analysis was not performed.
The bees were not dosed at 10× the maximum field application rate (MFAR) because the test substance did not form a homogenous dispersion at 194 g/L. The bees were dosed at the MFAR of 19.4 g/L instead. The protocol deviation had an effect on the outcome of the study. Instead of evaluation of the test substance at 10× MFAR, evaluation was determined at MFAR.

Summary

This study was designed to assess the acute oral toxicity potential of the microbial pest control agent (MPCA) test substance, *Beauveria bassiana* PPRI 5339 spore concentrate when administered to the honey bee at the rate of 19.4 g/L which was equivalent to $4.0 \times$ CFU/mL. This study cannot be used to support the registration of *Beauveria bassiana* strain PPRI 5339. A more recent study has been conducted and this is summarised under MA 8.3/07.

Materials and methods

Start of laboratory phase: October 2012

End of laboratory phase: October 2012

Test item

Name: *Beauveria bassiana* PPRI 5339 spore concentrate
Batch No.: S627 C2
Physical State: Light cream powder
Purity: $2.06 \times$ spores/g
Storage conditions: Refrigerated

Positive control

Name: Dimethoate
Dose: 0.01 mg/L

Test organism

Species: *Apis mellifera*
 Supplier: STILLMEADOW Inc.
 Life stage of supplied test bees: Young adults

Honey bees, *Apis mellifera*, divided into four groups – Group I – untreated (vehicle control), Group II – inactive *Beauveria bassiana* PPRI 5339 spore concentrate, Group III – *Beauveria bassiana* PPRI 5339 spore concentrate and Group IV – dimethoate (positive control). Each group consisted of 2 cages of 100 bees.

Bees in group III were dosed with the test item at 19.4 g/L. Given that the spore content of the test substance was $2.06 \times$ spores per g the concentration of the test solution was $4.0 \times$ CFU/mL. This is $200 \times$ the maximum field application rate (MFAR).

The bees were observed daily for pathogenicity, toxicity or mortality. Observations were made until mortality in the control group exceeded 20%.

Dose verification was performed by diluting 19.4 g of the test material in 1 L of a 50% sucrose solution. This was plated onto Rose Bengal Agar. The plates were incubated for 48 h at 28°C.

Findings

Results were evaluated by comparing corrected percent mortality between control group and treated group.

Mortality observations and percent mortality are presented in Table 8.3/01-1.

Table 8.3/01-1: Percent mortality summary for effect of *Beauveria bassiana* strain PPRI 5339 on honey bees

Day	Mortality (%)				p-value
	Group I Untreated	Group II Inactive	Group III Active	Group IV Dimethoate	
1	0.5 a	0.5 a	0.0 a	1.0 a	0.3813
2	1.0 a	5.0 ab	11.5 b	1.0 a	0.0112
3	2.0 a	51.5 b	69.0 c	1.0 a	<0.0001
4	6.0 a	81.5 b	97.5 c	4.0 a	<0.0001
5	7.5 a	94.5 b	100.0 b	8.5 a	<0.0001
6	10.0 a	100.0 b	100.0 b	19.5 a	<0.0001
7	14.0 a	100.0 b	100.0 b	26.5 c	<0.0001
8	17.5 a	100.0 b	100.0 b	38.5 c	<0.0001
9	17.5 a	100.0 b	100.0 b	43.5 c	<0.0001
10	24.5 a	100.0 b	100.0 b	52.5 c	<0.0001

Different letter within the same row indicate significance at $p < 0.05$

Mortality was 100% for bees fed the inactive test substance and by Day 5 for bees that were fed the active day substance. The study ended on Day 10 when mortality in the control group exceeded 20%.

Given that >50% mortality had occurred in the group treated with the inactive *Beauveria bassiana* by Day 3 and that 100% mortality had occurred by Day 6 this test is not considered to be reliable. Also, the pre-dose enumeration yielded counts of $8.5 \times$ CFU/mL. This is far lower

than the target dose of 4.0 x CFU/mL. It is therefore considered that this study is unacceptable for use in the authorisation process for *Beauveria bassiana* strain PPRI 5339.

Conclusions

This study cannot be used to support the registration of *Beauveria bassiana* strain PPRI 5339. for the following reasons: Given that the maximum concentration of substance applied in a greenhouse would be 2 x CFU/mL the verified dose in this study would have been 2350 x too low. Also, this study can be disregarded given that 51.5% mortality occurred after 3 days in the inactive control. Given that 1% mortality occurred for the toxic reference at the same assessment time it is clear that there was a major problem in the dosing of the bees with the inactive and the active microbial substance.

A more recent study has been conducted and this is summarised under MA, Point 8.3/07.

MA 8.3/02

Report: Younger C (2012). *Beauveria bassiana* PPRI 5339 spore concentrate. Honey Bee, *Apis mellifera* non-target microbial testing. Stillmeadow Inc., 12852 Park One Drive, Sugar Land TX 77478. Study Code: 16984-12

Guideline: OCSPP 885, 4380

GLP: Yes

Deviations: Characterization and stability information was not provided to the testing facility.

Summary

This study was designed to assess the acute toxicity potential of the microbial pest control agent (MPCA) test substance, *Beauveria bassiana* strain PPRI 5339 spore concentrate when administered to the honey bee at the application rate of 400 g/ha. The LD_{50} for the test item was determined to be greater than 16 days, and the LD_{10} was determined to be $>3.9 \times$ CFU/mL. However, mortality in the inactive control was unacceptably high meaning that this study cannot be used in the risk assessment. A summary of a repeat study is presented under point MA 8.3/05.

Materials and methods

Start of laboratory phase: December 2012

End of laboratory phase: December 2012

Test item

Name: *Beauveria bassiana* PPRI 5339 spore concentrate

Batch No.: S627 C2

Physical State: Light cream powder

Purity: $2.06 \times$ spores/g

Storage conditions: Refrigerated

Toxic reference

Name: Dimethoate

Dose: 0.1 mg/L

Test organism

Species: *Apis mellifera*
 Supplier: STILLMEADOW Inc.
 Lifestage of supplied test bees: Young adults

Honey bees, *Apis mellifera*, divided into four groups: untreated (vehicle control), inactive *Beauveria bassiana* PPRI 5339 spore concentrate, *Beauveria bassiana* PPRI 5339 spore concentrate and dimethoate (positive control). Each group consisted of 2 cages of 100 bees.

Bees in the group treated with the active spore concentration were dosed with 400 g MCPA/ha. To prepare this 1.57 mg of the test substance was suspended in 1 L of a 50% sucrose solution. Given that the spore content of the test substance was $2.06 \times$ spores per g the concentration of the test solution would nominally be $3.23 \times$ CFU/mL.

The bees were observed daily for pathogenicity, toxicity or mortality. Observations were made until mortality in the control group exceeded 20%.

Dose verification was performed by diluting 1 mL of the test solution in 10 mL with PBS. This was plated onto DRBC agar. The plates were incubated for 72 h at 28°C.

Results

Results were evaluated by comparing corrected percent mortality between control group and treated group.

Mortality observations and percent mortality are presented in Table 8.3/02-1.

Table 8.3/02-1: Daily mean mortality summary for effect of *Beauveria bassiana* strain PPRI 5339 on honey bees

Day	Mortality (%)				p-value
	Vehicle Control	Inactive	Active	Dimethoate	
0 (4 hours)	0.0	0.0	0.0	0.5	0.4789
1	0.0 a	0.0 a	0.0 a	1.0 a	0.4789
2	0.0 a	0.0 a	0.0 a	1.0 a	0.4789
3	0.5 a	0.5 a	1.0 a	1.0 a	0.8766
4	1.0 a	0.5 a	3.0 a	2.0 a	0.5607
5	1.5 a	1.5 a	6.5 a	2.5 a	0.6418
6	1.5 a	1.5 a	6.5 a	3.0 a	0.6369
7	2.5 a	2.0 a	6.5 a	3.5 a	0.7357
8	2.5 a	3.0 a	9.0 a	4.0 a	0.7177
9	2.5 a	2.5 a	10.0 a	5.0 a	0.6086
10	3.0 a	4.0 a	12.5 a	5.0 a	0.4316
11	5.0 a	4.5 a	16.5 a	15.0 a	0.4561
12	8.5 a	12.5 a	17.5 a	23.0 a	0.8116
13	11.5 a	21.0 a	19.0 a	30.0 a	0.8664
14	14.0 a	20.0 a	21.5 a	31.5 a	0.8671

15	19.0 a	22.5 a	24.0 a	42.0 a	0.7468
16	24.0 a	40.0 a	27.0 a	57.5 a	0.7487

Different letter within the same row indicate significance at $p < 0.05$

The study ended on Day 16 when the mortality in the control group exceeded 20%. There were no significant differences in mortality among the groups.

The pre-dose enumeration analysis of the active and inactive test substance dosing solutions resulted in counts of $3.9 \times \text{CFU/mL}$ and 0 CFU/mL , respectively. The maximum field application rate (MFAR) is $2 \times \text{CFU/mL}$. Therefore *Beauveria bassiana* strain PPRI 5339 was tested at a concentration approximately $500 \times$ lower than the MFAR.

Conclusions

The for the test item was determined to be greater than 16 days, and the was determined to be $>3.9 \times \text{CFU/mL}$. However, mortality in the inactive control was unacceptably high meaning that this study cannot be used in the risk assessment. This study cannot be used to support the registration of *Beauveria bassiana* strain PPRI 5339 for the following reasons: The concentration of the pre-dose enumeration analysis of the active test substance dosing solutions resulted in counts of $3.9 \times \text{CFU/mL}$. This is $500 \times$ lower than the intended application rate of $2 \times \text{CFU/mL}$ PPRI 5339.

A summary of a repeat study is presented under point MA 8.3/07

MA 8.3/03

Report: Whittaker M (2014), Effects of *Beauveria bassiana* PPRI 5339 on the honeybee, *Apis mellifera*, in an acute oral and contact toxicity pre-test. APIS, Knaresborough Technology Park, Manse Lane, Knaresborough, North Yorkshire, HG5 8LF. Study No.: APIS-BASF-NGLP-001

Guidelines: Modification of OECD 213 and 214, (OECD, 1998)

GLP: No

Deviations: None

Summary

The aim of this preliminary test was to determine:

- The suitability of the test chamber and feeding tubes and the control mortality achievable in a 10-day test
- The feasibility of application of $100 \mu\text{L}$ as a topical dose, as per guideline for substances of presumed low toxicity
- The mortality following oral and contact exposure to the microbial pest control agent *Beauveria bassiana* strain PPRI 5339 at the Maximum Hazard Concentration (MHC), in terms of (above or below the MHC) and LT_{50} (shorter or longer than the 10 day study period).

Everything was acceptable. Also at the MHC *Beauveria bassiana* strain PPRI 5339 exhibited limited infectivity to honey bee. The LT_{50} was >10 days and the was $>\text{MHC}$.

Materials and methods

Start of laboratory phase: August 2013
End of laboratory phase: August 2013

Test item

Name: *Beauveria bassiana* PPRI 5339
Batch No.: S826
Physical State: Off-white powder
Purity: 1 × CFU/g nominal
Storage conditions: Refrigerated

Toxic reference

Name: Perfekthion (dimethoate)
Batch No.: 0001040432
Expiry date: August 2015
Doses: 0.05 (low), 0.10 (medium) and 0.15 µg/bee

Test organism

Species: *Apis mellifera*
Supplier: Leeds Beekeepers Association
Life stage of supplied test bees: Mixed, young workers

Ten young worker honeybees (*Apis mellifera*) were placed into each of the three test chambers per treatment. The bees were subject to treatment with either a water control solution (contact test), sucrose control (oral test) or *Beauveria bassiana* strain PPRI 5339.

The test was conducted in cylindrical Perspex tubes (length 180 mm, internal diameter 45 mm). A single hole (9 mm diameter) had been drilled halfway along the tube. This served as a port for the feeding tubes.

For the contact test, a 100 µL droplet of *Beauveria bassiana* PPRI 5339 was dosed onto the dorsal surface of the thorax of each bee at the Maximum Hazard Concentration (MHC) of the MPCA. For preparation of this, 0.20 g of MPCA was diluted in 100 g deionised water. Since it is normal practice to apply a 1 µL droplet in contact toxicity tests the test solution was diluted a further 100-fold in order to compensate for the application of a 100 µL droplet.

For the oral test, 200 µL was administered to each cage. This was replaced with untreated 50% sucrose solution after all of the dose had been consumed. The assumption made was that equal feeding between the bees would result in each bee receiving 20 µL.

A single mortality assessment was made 10 days after treatment. The bees in each chamber were classified as alive, affected, moribund or dead.

Results

Suitability of the test chamber and feeding tubes and control mortality over 10 days

The test chambers and feeding tubes were considered to be fit for purpose. Also, control mortality was 20% in the sucrose control and 13.3% in the water control. These were considered to be acceptable.

Feasibility of applying a 100 µL droplet for the contact dose

Application of a 100 µL droplet represented the maximum realistic volume that could be applied before run-off. Control mortality was lower than in the oral control group, suggesting that the higher application rate had no effect on bee survival.

Mortality following oral and contact exposure to the MCPA *Beauveria bassiana* PPRI 5339 applied at the MHC

Mortality was 30% and 26.7% for application via the oral and contact routes of exposure, respectively. As mortality was <50% the oral and contact values were greater than the MHC and the was greater than the 10-day study duration.

Conclusions

In a preliminary test test chamber and feeding tube suitability, control mortality achievable in a 10-day test, feasibility of application of 100 µL as a topical dose and mortality following oral and contact exposure to the microbial pest control agent *Beauveria bassiana* strain PPRI 5339 at the MHC were evaluated.

Everything was acceptable. Also at the MHC *Beauveria bassiana* strain PPRI 5339 exhibited limited infectivity to honey bee. The LT50 was >10 days and the was >MHC.

MA 8.3/04

Report: Whittaker M (2013), Effects of *Beauveria bassiana* PPRI 5339 on the bumblebee, *Bombus terrestris*, in an acute contact toxicity test. APIS, Knaresborough Technology Park, Manse Lane, Knaresborough, North Yorkshire, HG5 8LF. Study No.: APIS-BASF-004

Guidelines: Modification of OECD 214, (OECD, 1998)
OPPTS 885.4340 (EPA, 1996)
OPPTS 885.4380 (EPA, 1996)

GLP: Yes

Deviations: None

Summary

The study was conducted to determine the median lethal time () and following contact exposure of the microbial pest control agent (MPCA) *Beauveria bassiana* strain PPRI 5339 on the bumblebee, *Bombus terrestris*. The for the test item >10 days. The was >3.1 × CFU/bee.

Materials and methods

Start of laboratory phase: October 2013

End of laboratory phase: October 2013

Test item

Name: *Beauveria bassiana* PPRI 5339
Batch No.: S826
Physical State: Off-white powder
Purity: 1 × CFU/g nominal
Storage conditions: Refrigerated

Toxic reference

Name: Perfekthion (dimethoate)
Batch No.: 0001040432
Expiry date: August 2015
Doses: 0.05 (low), 0.10 (medium) and 0.15 µg/bee

Test organism

Species: *Bombus terrestris*
Supplier: Koppert Biological Systems, Haverhill, Suffolk, UK
Life stage of supplied test bees: Mixed (Tripol hive)

Five young worker bumblebees (*Bombus terrestris*) were placed into each of the six test chambers per treatment. The bees were subject to treatment with either a water control solution, Tween 80 adjuvant, inactive *Beauveria bassiana* strain PPRI 5339, active *Beauveria bassiana* strain PPRI 5339 or dimethoate positive control (at low, medium or high concentrations).

The test was conducted in cylindrical Perspex tubes (length 180 mm, internal diameter 45 mm). A single hole (9 mm diameter) had been drilled halfway along the tube. This served as a port for the feeding tubes.

A 100 µL droplet of *Beauveria bassiana* PPRI 5339 was dosed onto the dorsal surface of the thorax of each bee at the Maximum Hazard Concentration (MHC) of the MPCA. For preparation of this, 0.202 g of MPCA was diluted in 100.02 g deionised water. Since it is normal practice to apply a 1 µL droplet in contact toxicity tests the test solution was diluted a further 100-fold in order to compensate for the application of a 100 µL droplet. The inactive MCPA was applied at the same dose as for the active *Beauveria bassiana*.

Dose verification was performed by diluting the spray solution in a logarithmic scale and plating 100 µL from each dilution onto Rose Bengal Agar. The plates were incubated for 96 h.

Mortality assessments were made approximately 4-6 hours after treatment and every 24 hours thereafter, for ten days. At each observation, the bees in each chamber were classified as alive, affected, moribund or dead.

Bees that died during the study were removed from the test chamber and placed in individual Petri dishes. These dishes were placed in an incubator to allow for the growth of any infective organisms. These were subsequently plated onto Rose Bengal Agar to confirm their identity.

At the end of the study, three bees from each test chamber were subjected to autopsy. External signs of infection were noted before internal examination of the head and abdomen.

Results

One bee died in the MPCA treatment during the study which showed no over signs of infections after post-mortem incubation or any sign of infection at autopsy.

The mortality per treatment group at 10 days after treatment is shown in Table 8.3/03-1.

Table 8.3/04-1: Mortality of bumble bees (*Bombus terrestris*) by treatment group at 10 days after treatment with *Beauveria bassiana* strain PPRI 5339

Treatment	Observations at 10 days after treatment (30 bees per treatment)			
	Alive	Affected	Moribund	Dead
Water control	28	0	0	2
Adjuvant	27	0	0	3
Inactive MPCA	26	0	0	4
MPCA	29	0	0	1
Toxic Reference (Low)	0	0	0	30
Toxic Reference (Medium)	0	0	0	30
Toxic Reference (High)	0	0	0	30

Dose verification revealed that the concentration of MPCA applied to the bees was $3.1 \times \text{CFU/mL}$ ($3.1 \times \text{CFU}/\mu\text{L}$).

For application of *Beauveria bassiana* strain PPRI 5339 at the maximum application rate of 1.0 x /ha (in the minimum application volume of 500 L) and assuming that each foraging bee would encounter a 1 μL droplet, then the maximum theoretical amount encountered per bee would be 2 x CFU.

Since each bee in this test was treated with a 100 μL droplet then the dose would have been $3.1 \times \text{CFU}/\text{bee}$ which represents 16 times the maximum field rate. As the test item was applied at a single dose representing the Maximum Hazard Concentration (16 times the maximum field rate), the calculation of an is not appropriate.

No infectivity or pathogenicity was recorded.

As mortality at 10 days in the MPCA was 1 out of 30 bees (3.3%) the is greater than the MHC of $3.1 \times \text{CFU}/\text{bee}$. The is also greater than 10 days.

Conclusions

At the Maximum Hazard Concentration, *Beauveria bassiana* PPRI 5339 exhibited no infectivity or pathogenicity to bumblebees. The for the test item >10 days. The was $>3.1 \times \text{CFU}/\text{bee}$.

MA 8.3/05

Report: Whittaker M (2013), Effects of *Beauveria bassiana* PPRI 5339 on the bumblebee, *Bombus terrestris*, in an acute oral toxicity test. APIS, Knaresborough Technology Park, Manse Lane, Knaresborough, North Yorkshire, HG5 8LF. Study No.: APIS-BASF-005

Guidelines: Modification of OECD 213, (OECD, 1998)
OPPTS 885.4340 (EPA, 1996)
OPPTS 885.4380 (EPA, 1996)

GLP: Yes

Deviations: None

Aim of the study

The study was conducted to determine the median lethal time () and following oral exposure of the microbial pest control agent (MPCA) *Beauveria bassiana* strain PPRI 5339 on the bumblebee, *Bombus terrestris*. The for the test item >10 days. The was >2.9 × CFU/bee.

Materials and methods

Start of laboratory phase: October 2013

End of laboratory phase: October 2013

Test item

Name: *Beauveria bassiana* PPRI 5339

Batch No.: S826

Physical State: Off-white powder

Purity: 1 × CFU/g nominal

Storage conditions: Refrigerated

Toxic reference

Name: Perfekthion (dimethoate)

Batch No.: 0001040432

Expiry date: August 2015

Doses: 0.05 (low), 0.15 (medium) and 0.50 µg/bee

Test organism

Species: *Bombus terrestris*

Supplier: Koppert Biological Systems, Haverhill, Suffolk, UK

Lifestage of supplied test bees: Mixed (Tripol hive)

Five young worker bumblebees (*Bombus terrestris*) were placed into each of the six test chambers per treatment. The bees were subject to treatment with either a sucrose control solution, Tween 80 adjuvant, inactive *Beauveria bassiana* strain PPRI 5339, active *Beauveria bassiana* strain PPRI 5339 or dimethoate positive control (at low, medium or high concentrations).

The test was conducted in cylindrical Perspex tubes (length 180 mm, internal diameter 45 mm). A single hole (9 mm diameter) had been drilled halfway along the tube. This served as a port for the feeding tubes.

The bees were dosed with *Beauveria bassiana* strain PPRI 5339 at the Maximum Hazard Concentration (MHC) of the MPCA. For preparation of this, 0.202 g of MPCA was diluted in 100.02 g 50% sucrose solution prepared in deionised water. 500 µL of test solution was provided and this was completely consumed within a 4-6 hour period. It is assumed that the bees shared the dose equally meaning that each bee consumed 100 µL of a $2.9 \times$ CFU/mL solution of MPCA. This represented a concentration of $2 \times$ CFU/mL. The inactive MPCA was applied at the same dose as for the active *Beauveria bassiana*.

Dose verification was performed by diluting the spray solution in a logarithmic scale and plating 100 µL from each dilution onto Rose Bengal Agar. The plates were incubated for 96 h.

Mortality assessments were made approximately 4-6 hours after treatment and every 24 hours thereafter, for ten days. At each observation, the bees in each chamber were classified as alive, affected, moribund or dead.

Bees that died during the study were removed from the test chamber and placed in individual petri dishes. These dishes were placed in an incubator to allow for the growth of any infective organisms. These were subsequently plated onto Rose Bengal Agar to confirm their identity.

At the end of the study, three bees from each test chamber were subjected to autopsy. External signs of infection were noted before internal examination of the head and abdomen.

Results

Two bees died in the MPCA treatment during the study, neither of which showed no over signs of infections after post-mortem incubation or any sign of infection at autopsy. A further 16 bees were euthanized at study termination, none of which showed any signs of infection.

The mortality per treatment group at 10 days after treatment is shown in Table 8.3/04-1.

Table 8.3/05-1: Mortality of bumble bees (*Bombus terrestris*) by treatment group at 10 days after treatment with *Beauveria bassiana* strain PPRI 5339

Treatment	Observations at 10 days after treatment (30 bees per treatment)			
	Alive	Affected	Moribund	Dead
Sucrose control	27	0	0	3
Adjuvant	28	0	0	2
Inactive MPCA	28	0	0	2
MPCA	28	0	0	2
Toxic Reference (Low)	0	0	0	30

Toxic Reference (Medium)	0	0	0	30
Toxic Reference (High)	0	0	0	30

Dose verification revealed that the concentration of MPCA in the oral solution was $2.9 \times$ CFU/mL ($2.9 \times$ CFU/ μ L).

For application of *Beauveria bassiana* strain PPRI 5339 at the maximum application rate of 1.0 x /ha (in the minimum application volume of 500 L), and assuming that each foraging bee would consume 100 μ L of nectar, the maximum amount consumed per bee would theoretically be 2 x CFU.

Since each bee in this test was assumed to have consumed 100 μ L then the dose consumed would be equivalent to $2.9 \times$ CFU/bee, which represents 15 times the maximum field rate. As the test item was applied at a single dose representing the Maximum Hazard Concentration (15 times the maximum field rate), the calculation of an is not appropriate.

Conclusions

At the Maximum Hazard Concentration, *Beauveria bassiana* PPRI 5339 exhibited no infectivity or pathogenicity to bumblebees. The for the test item >10 days. The was > $2.9 \times$ CFU/bee.

MA 8.3/06

Report: Whittaker M (2013), Effects of *Beauveria bassiana* PPRI 5339 on the honeybee, *Apis mellifera*, in an acute contact toxicity test. APIS, Knaresborough Technology Park, Manse Lane, Knaresborough, North Yorkshire, HG5 8LF. Study No.: APIS-BASF-001

Guidelines: Modification of OECD 214, (OECD, 1998)
OPPTS 885.4340 (EPA, 1996)
OPPTS 885.4380 (EPA, 1996)

GLP: Yes

Deviations: The temperature in the growth chamber exceeded 27 °C for extended periods, reaching 31 °C on two occasions.

Summary

The study was conducted to determine the median lethal time () following contact exposure of the microbial pest control agent (MPCA) *Beauveria bassiana* strain PPRI 5339 on the honeybee, *Apis mellifera*. The for the test item >10 days. The was > $2.9 \times$ CFU/bee.

Materials and methods

Start of laboratory phase: October 2013

End of laboratory phase: October 2013

Test Item

Name: *Beauveria bassiana* PPRI 5339
Batch No.: S826
Physical State: Off-white powder
Purity: 1 x CFU/g nominal
Storage conditions: Refrigerated

Toxic reference

Name: Perfekthion (dimethoate)
Batch No.: 0001040432
Expiry date: August 2015
Doses: 0.05 (low), 0.15 (medium) and 0.50 µg/bee

Test organism

Species: *Apis mellifera*
Supplier: Arcadian Apiaries Ltd., Harrogate, UK
Lifestage of supplied test bees: Young adults

Ten young worker honeybees (*Apis mellifera*) were placed into each of the three test chambers per treatment. The bees were subject to treatment with either a water control solution, Tween 80 adjuvant, inactive *Beauveria bassiana* strain PPRI 5339, active *Beauveria bassiana* strain PPRI 5339 or dimethoate positive control (at low, medium or high concentrations).

The test was conducted in cylindrical Perspex tubes (length 180 mm, internal diameter 45 mm). A single hole (9 mm diameter) had been drilled halfway along the tube. This served as a port for the feeding tubes.

A 100 µL droplet of *Beauveria bassiana* PPRI 5339 was dosed onto the dorsal surface of the thorax of each bee at the Maximum Hazard Concentration (MHC) of the MPCA. For preparation of this, 0.20 g of MPCA was diluted in 100 g deionised water. Since it is normal practice to apply a 1 µL droplet in contact toxicity tests the test solution was diluted a further 100-fold in order to compensate for the application of a 100 µL droplet. The inactive MPCA was applied at the same dose as for the active *Beauveria bassiana*.

Dose verification was performed by diluting the spray solution in a logarithmic scale and plating 100 µL from each dilution onto Rose Bengal Agar. The plates were incubated for 96 h.

Mortality assessments were made approximately 4-6 hours after treatment and every 24 hours thereafter, for ten days. At each observation, the bees in each chamber were classified as alive, affected, moribund or dead.

Bees that died during the study were removed from the test chamber and placed in individual petri dishes. These dishes were placed in an incubator to allow for the growth of any infective organisms. These were subsequently plated onto Rose Bengal Agar to confirm their identity.

At the end of the study, three bees from each test chamber were subjected to autopsy. External signs of infection were noted before internal examination of the head and abdomen.

Results

Eight bees died in the MPCA treatment during the study, neither of which showed no over signs of infections after post-mortem incubation. Seven of these bees and two surviving bees, euthanized at study termination, were autopsied, and showed no signs of infection.

The mortality per treatment group at 10 days after treatment is shown in Table 8.3/05-1.

Table 8.3/06-1: Mortality of honey bee (*Apis mellifera*) by treatment group at 10 days after treatment with *Beauveria bassiana* strain PPRI 5339

Treatment	Observations at 10 days after treatment (30 bees per treatment)			
	Alive	Affected	Moribund	Dead
Water control	26	0	0	4
Adjuvant	11	0	0	19
Inactive MPCA	21	0	0	9
MPCA	22	0	0	8
Toxic Reference (Low)	10	0	0	20
Toxic Reference (Medium)	4	0	0	26
Toxic Reference (High)	0	0	0	30

Dose verification revealed that the concentration of MPCA applied to the bees was $2.9 \times$ CFU/mL ($2.9 \times$ CFU/ μ L).

For application of *Beauveria bassiana* strain PPRI 5339 at the maximum application rate of 1.0 x /ha (in the minimum application volume of 500 L), and assuming that each foraging bee would encounter a 1 μ L droplet, then the maximum theoretical amount encountered per bee would be 2 x CFU.

Since each bee in this current study was treated with a 100 μ L droplet then the dose would have been $2.9 \times$ CFU/bee which represents 15 times the maximum field rate. As the test item was applied at a single dose representing the Maximum Hazard Concentration (15 times the maximum field rate), the calculation of an is not appropriate.

As the test item was applied at a single dose representing the Maximum Hazard Concentration (15 times the maximum field rate), the calculation of an is not appropriate. As mortality at 10 days in the MPCA was only 27% the is greater than the MHC. The is also greater than 10 days.

Conclusions

At the Maximum Hazard Concentration, *Beauveria bassiana* strain PPRI 5339 exhibited limited infectivity to honey bees. The for the test item >10 days. The was $>2.9 \times$ CFU/bee.

MA 8.3/07

Report: Whittaker M (2013), Effects of *Beauveria bassiana* PPRI 5339 on the honeybee, *Apis mellifera*, in an acute oral toxicity test. APIS, Knaresborough Technology

Park, Manse Lane, Knaresborough, North Yorkshire, HG5 8LF. Study No.: APIS-BASF-002

Guidelines: Modification of OECD 214, (OECD, 1998)
OPPTS 885.4340 (EPA, 1996)
OPPTS 885.4380 (EPA, 1996)

GLP: Yes

Deviations: The temperature in the growth chamber exceeded 27 °C for extended periods, reaching 31 °C on two occasions

Summary

The study was conducted to determine the median lethal time () following oral exposure of the microbial pest control agent (MPCA) *Beauveria bassiana* strain PPRI 5339 on the honeybee, *Apis mellifera*. At the Maximum Hazard Concentration, *Beauveria bassiana* PPRI 5339 exhibited limited infectivity to honey bees. The for the test item >10 days. The was >9.57 × CFU/bee.

Materials and methods

Start of laboratory phase: September 2013

End of laboratory phase: September 2013

Test Item

Physical State: Off-white powder
Purity: 1 × CFU/g nominal
Storage conditions: Refrigerated

Toxic reference

Name: Perfekthion (dimethoate)
Batch No.: 0001040432
Expiry date: August 2015
Doses: 0.05 (low), 0.15 (medium) and 0.50 µg/bee

Test organism

Species: *Apis mellifera*
Supplier: Arcadian Apiaries Ltd., Harrogate, UK
Lifestage of supplied test bees: Young adults

Ten young worker honeybees (*Apis mellifera*) were placed into each of the three test chambers per treatment. The bees were subject to treatment with either a sucrose control solution, Tween 80 adjuvant, inactive *Beauveria bassiana* strain PPRI 5339, active *Beauveria bassiana* strain PPRI 5339 or dimethoate positive control (at low, medium or high concentrations).

The test was conducted in cylindrical Perspex tubes (length 180 mm, internal diameter 45 mm). A single hole (9 mm diameter) had been drilled halfway along the tube. This served as a port for the feeding tubes.

The bees were dosed with *Beauveria bassiana* PPRI 5339 at the MHC of the MPCA. For preparation of this, 0.20 g of MPCA was diluted in 100 g 50% sucrose solution prepared in deionised water. The inactive MCPA was applied at the same dose as for the active *Beauveria bassiana*. For dosing, 200 µL was administered to each cage. This was replaced with untreated 50% sucrose solution after all of the dose had been consumed. The assumption made was that equal feeding between the bees would result in each bee receiving 20 µL.

Dose verification was performed by diluting the spray solution in a logarithmic scale and plating 100 µL from each dilution onto Rose Bengal Agar. The plates were incubated for 96 h.

Mortality assessments were made approximately 4-6 hours after treatment and every 24 hours thereafter, for ten days. At each observation, the bees in each chamber were classified as alive, affected, moribund or dead.

Bees that died during the study were removed from the test chamber and placed in individual petri dishes. These dishes were placed in an incubator to allow for the growth of any infective organisms. These were subsequently plated onto Rose Bengal Agar to confirm their identity.

At the end of the study, three bees from each test chamber were subjected to autopsy. External signs of infection were noted before internal examination of the head and abdomen.

Results

Nine bees died in the MPCA treatment during the study, of which five showed signs of infection in the form of profuse fluffy white fungal growth over the cadaver after post-mortem incubation. This was subsequently identified as *Beauveria bassiana* in all cases after culturing on Rose Bengal Agar.

The remaining four bees that died during the study showed no signs of infection at autopsy. Similarly, none of the surviving bees euthanized at study termination showed any signs of infection.

The mortality per treatment group at 10 days after treatment is shown in Table 8.3/06-1.

Table 8.3/07-1: Mortality of honey bees (*Apis mellifera*) by treatment group at 10 days after treatment with *Beauveria bassiana* strain PPRI 5339

Treatment	Observations at 10 days after treatment (30 bees per treatment)			
	Alive	Affected	Moribund	Dead
Sucrose control	24	0	0	6
Adjuvant	20	0	0	10
Inactive MPCA	28	0	0	2
MPCA	21	0	0	9
Toxic Reference (Low)	0	0	0	30
Toxic Reference (Medium)	0	0	0	30

Toxic Reference (High)	0	0	0	30
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The mean amount of solution consumed after 4 – 6 hours was 0.33 g per cage of 10 bees. If it is assumed that the bees shared the dose equally then each bee consumed 0.033 g.

Dose verification revealed that the concentration of MPCA in the oral solution was $2.9 \times$ CFU/mL.

For application of *Beauveria bassiana* strain PPRI 5339 at the maximum application rate of 1.0 x /ha (in the minimum application volume of 500 L), and assuming that each foraging bee would consume 20 µL of nectar, the maximum amount consumed per bee would theoretically be 4 x CFU.

Since each bee in this study was assumed to have consumed 0.033 g, then the dose consumed would be equivalent to $9.57 \times$ CFU/bee, which represents 24 times the maximum field rate. As the test item was applied at a single dose representing the Maximum Hazard Concentration (24 times the maximum field rate), the calculation of an is not appropriate.

Conclusions

At the Maximum Hazard Concentration, *Beauveria bassiana* PPRI 5339 exhibited limited infectivity to honey bees. The for the test item >10 days. The was $>9.57 \times$ CFU/bee.

From literature:

MA 8.3/08

Citation: Meikle, WG, Mercadier, G, Holst, N, Girod, V (2008). Impact of two treatments of a formulation of *Beauveria bassiana* (Deuteromycota: Hyphomycetes) conidia on Varroa mites (Acari: Varroidae) and on honeybee (Hymenoptera: Apidae) colony health. *Experimental and Applied Acarology*, 46(1-4): 105-117

Guidelines: None

GLP: No

Summary

Bee colonies in southern France were treated with conidia (asexual spores) from two strains of *Beauveria bassiana*. Objectives were to evaluate treatment effect on colony weight, adult bee mass, capped brood. Treatments included conidia formulated with either carnauba or candelilla wax powder, candelilla wax powder alone, or control; in two treatment groups, formulation was applied a second time after one week. Treatment did not affect colony health. The number of fungal propagules on the bees themselves remained elevated for approximately 14 days after the second treatment.

Materials and methods

Four formulations were prepared:

- carnauba wax powder and conidia from *Beauveria bassiana* isolate 05002

(“Bb05002 + carnauba”);

- carnauba wax powder and conidia from *Beauveria bassiana* isolate GHA (“BbGHA + carnauba”);
- candelilla wax powder and 05002 conidia (“Bb05002 + candelilla”);
- and candelilla wax powder alone (“candelilla alone”).

The per colony dose of Bb05002 + carnauba and BbGHA + carnauba consisted of 1.0 g conidia of the respective isolate mixed with 9.0 g carnauba wax powder and 0.05 g hydrated silica as a flow agent. The per colony dose of Bb05002 + candelilla consisted of 1.0 g conidia of isolate 05002 mixed with 9.0 g candelilla wax powder and 0.05 g hydrated silica. The per colony dose of candelilla alone consisted of 9.0 g candelilla wax powder and 0.05 g silica. The density of colony-forming units (CFU) per g formulation was determined at the time of colony treatment by plating three sub-samples of the formulation diluted in distilled water and Tween 80 onto potato-dextrose agar, and counting the number of colonies 96 h after plating.

Twenty six bee colonies were used for the field experiment. Each colony comprised a 10-frame wooden Langstroth brood box.

Five colonies were selected for each treatment group except the untreated control, which had 6. For each colony treatment, a plastic laboratory wash was filled with a single dose of preparation, the hive lid and super removed, the formulation blown between all brood box frames by squeezing the wash bottle, and the super and lid replaced.

To calculate colony and adult bee weight, hive weight was divided into a “non-colony” part, consisting of the hive pieces, e.g., brood box, lids, super, hive base, and 10 empty frames with foundation comb, and the “colony” part, consisting of the adult bees, brood, honey, pollen and wax (other than foundation comb). Adult bee weight was calculated as the difference between the sum of the weights of all the hive parts and the observed hive weight. The non-colony weight was calculated as the total weight of all the hive parts except brood box frames, plus the weight of 10 empty frames, or approximately 2.87 kg. Colony weight was calculated by subtracting the non-colony component from the total hive weight. The area of sealed brood and sealed honey per frame was estimated from photographs. Brood areas were inspected closely for any signs of fungal infection. Colony entrances were inspected for unusually large numbers of dead bees.

On several occasions throughout May and June, samples of approximately 15 bees were collected from within each hive in order to evaluate the number CFUs on them. Subsamples of the bees were placed in a 50 mL plastic centrifuge tube and vortexed for 3 min. in 10 mL of a 0.1% aqueous solution of Tween 80. Aliquots of 20 and 100 μ L of the resulting suspension from each subsample were spread onto each of three Petri dishes containing potato dextrose agar with chloramphenicol (0.4 g/L). The dishes were incubated for at least 14 days at 23°C, and the number of *Beauveria bassiana* CFU were counted in the plates with 20 μ L of solution; when CFU densities became low, CFU were counted on the 100 μ L plates.

Findings

CFU density at time of treatment was $3.70 \times$ CFU/g for the Bb05002 + carnauba formulation,

$1.79 \times$ CFU/g for the Bb05002 + candelilla formulation and $1.72 \times$ CFU/g for the BbGHA + carnauba formulation.

No negative effect of application of entomopathogenic fungi on colony health, measured as the colony growth rate, total adult bee weight, surface areas of capped brood, and colony survivorship was observed. Colony growth among all groups was lowest immediately after application, but this was likely to be due to food consumption prior to a nectar flow. Colony growth increased among all groups thereafter. No treatment differences were observed in either total adult weight change or changes in the amounts of sealed brood or honey.

Conclusions

Bee hives were treated with two strains of *Beauveria bassiana* at application rates ranging from $1.72 \times$ CFU/g to $3.70 \times$ CFU/g. No negative effects were observed on colony health, measured as the colony growth rate, total adult bee weight, surface areas of capped brood, and colony survivorship.

MA 8.3/09

Citation: Meikle WG, Mercadier G, Holst N, Nansen C & Girod V (2008). Impact of a treatment of *Beauveria bassiana* (Deuteromycota: Hyphomycetes) on honeybee (*Apis mellifera*) colony health and on Varroa destructor mites (Acari : Varroidae). *Adipologie*, 39(2): 247-259

Guidelines: None

GLP: No

Summary

Bee colonies were treated in southern France with conidia of a *Beauveria bassiana* isolate collected from *Varroa* mites in the region. Objectives were to evaluate treatment effect on colony weight, adult bee mass, and capped brood and honey, and on *Varroa* fall onto sticky boards. Treatments included conidia formulated with either wax powder or wheat flour, flour alone, or control. Treatment did not affect colony health.

Materials and methods

Two field experiments were described in this paper. One of which concentrated on evaluation of any detrimental effects to treated bee colonies. The other evaluated the effects of *Beauveria bassiana* on *Varroa destructor*. The remainder of this summary will focus on the effects of *Beauveria bassiana* on colony health.

Three formulations were prepared prior to treatment:

- wax powder and conidia (“powder + conidia”);
- flour and conidia (“flour + conidia”);
- and flour alone.

The per colony dose of powder + conidia consisted of 1.0 g Bb05002 conidia mixed with 9.0 g

Entostator powder, a refined, electrostatically-chargeable carnauba wax powder and 0.05 g hydrated silica. The per colony dose of flour + conidia consisted of 1.0 g Bb05002 conidia mixed with 9.0 g commercially-prepared wheat flour and 0.05 g silica prepared on 18 May. The dose per colony of flour alone was 10.0 g wheat flour mixed with 0.05 g silica. The density of colony-forming units (CFU) per g formulation was determined at the time of colony treatment by plating three sub-samples of the formulation diluted in distilled water and Tween 80 onto potato-dextrose agar, and counting the number of colonies 96 h after plating.

The bee colonies were housed in 10-frame, wooden Dadant brood boxes (56 L capacity) with telescoping lids and with screens underneath the frames.

Prior to treatment each hive was weighed. After weighing, each hive was opened, and each hive part (i.e. brood box, lids, colony base, and frames after shaking them free of bees) was weighed using a smaller portable electronic balance. Digital photographs were taken of each side of each. Brood areas were inspected closely for any signs fungal infection. The hive was then reassembled, and one super containing 9 frames with wax foundation was weighed and placed on top of each colony. Hives were then weighed in their entirety once per week thereafter until the end of observations for a total of 6 sampling occasions. The brood box frames were again weighed individually, following the same procedure, and the super was also weighed.

For each colony treatment, a plastic laboratory wash bottle was filled with a single dose of preparation, the hive lid removed, the formulation blown between all the frames in the brood box by squeezing the wash bottle, and the lid replaced.

To calculate colony and adult bee weight, hive weight was divided into a “non-colony” part, consisting of the hive pieces, e.g., brood box, lids, super, hive base, and 10 empty frames with foundation comb, and the “colony” part, consisting of the adult bees, brood, honey, pollen and wax (other than foundation comb). Adult bee weight was calculated as the difference between the sum of the weights of all the hive parts and the observed hive weight. The non-colony weight was calculated as the total weight of all the hive parts except brood box frames, plus the weight of 10 empty frames. The average weight of an empty frame was estimated by weighing 22 frames with only foundation comb. Colony weight was calculated by subtracting the non-colony component from the total hive weight. Colony entrances were inspected for unusually large numbers of dead bees.

Findings

CFU density at time of treatment was $1.96 \times$ CFU/g for the powder + conidia formulation, and $6.33 \times$ CFU/g for the flour + conidia formulation.

No negative effect of application of entomopathogenic fungi on colony health, measured as the colony growth rate, total adult bee weight, surface areas of capped brood and honey, and colony survivorship was observed. Colony growth among all groups was lowest immediately after application, but this was likely due to food consumption prior to a nectar flow. Colony growth increased among all groups thereafter. No treatment differences were observed in either total adult weight change or changes in the amounts of sealed brood or honey.

Table 8.3/09-1 shows that there were no significant differences between the control hives and the colonies treated with *Beauveria bassiana*. In fact, the total adult weight was significantly higher in the powder + conidia treatment at both observation periods. This was also the case for the

sealed brood area for the first observation period.

Table 8.3/09-1: Effects of *Beauveria bassiana* on various hive parameters

Variable	Treatment	N	23 May		27 June		Daily <i>r</i>
				s.d.		s.d.	
Total adult weight	Powder + conidia	5	3.301 ab	0.899	4.773 a	0.954	0.0105
	Flour + conidia	5	2.805 b	0.575	4.245 ab	0.535	0.0118
	Flour alone	4	4.305 a	0.513	4.711 ab	0.866	0.0026
	Control	7	2.161b	0.865	3.242 b	1.083	0.0116
Sealed brood surface area	Powder + conidia	5	4532 a	551	3712 a	732	-0.0057
	Flour + conidia	5	3721 ab	982	3665 a	596	-0.0004
	Flour alone	4	4447 a	548	3790 a	587	-0.0046
	Control	7	2867 b	1105	2812 a	1018	-0.0006
Sealed honey surface area	Powder + conidia	5	4846 a	1800	5489 a	1099	0.0036
	Flour + conidia	5	2836 a	1750	3593 a	1870	0.0068
	Flour alone	4	4535 a	849	6009 a	1545	0.0080
	Control	7	4516 a	1431	5203 a	2524	0.0040

¹ Averages within a variable and within a date followed by different letters are significantly different using Tukey's HSD at $P < 0.05$ /

Conclusion

Bee hives were treated with *Beauveria bassiana* at application rates of $1.96 \times$ and $6.33 \times$ CFU/g. No negative effects were observed on colony health, measured as the colony growth rate, total adult bee weight, surface areas of capped brood, and colony survivorship.

MA 8.3/10

Citation: Mommaerts, V, Put, K, Smaghe, G (2011) *Bombus terrestris* as pollinator-and-vector to suppress *Botrytis cinerea* in greenhouse strawberry. *Pest Management Science*, 67(9): 1069-1075.

Guidelines: None

GLP: No

Summary

A study was designed to investigate the capacity of *Bombus terrestris* as a pollinator and vector of a biofungicide formulation in greenhouse strawberry flowers. Exposure of bumblebees to the MCA caused no adverse effects on bumblebee workers, with no loss of survival or impairment of flight activity of the workers during the 4 week flowering period.

Materials and Methods

Bombus terrestris bumblebee hives obtained from a mass rearing program at Biobest NV (Westerlo, Belgium) were used for the greenhouse experiment. Each hive contained a queen, her brood and a minimum of 75 workers.

The biofungicide Prestop-Mix, based on *G. catenulatum* J1446 (now *C. rosea* f. *catenulata* J1446) [powder formulation containing – colony-forming units (CFU)/g and developed to be

delivered by pollinators], was used in this study as a model product for vectoring by bumblebees to suppress the plant pathogen *B. cinerea*. Before use, the numbers of CFU of *C. rosea* f. *catenulata* J1446 were determined through plating out on potato dextrose agar (PDA) medium.

The experiment was conducted in a greenhouse compartment of 24 × 63 × 3.75 m containing four fine-meshed tents (width 6 m, length 15 m, height 2 m) in Westerlo (Belgium). In each tent, a total of 100 strawberry plants were placed when flower buds appeared.

The flight activity was determined for each hive by counting the numbers of bumblebee workers (foragers) flying in and out over a 30 min period each day at 9 a.m. The foraging intensity of the bumblebee workers was evaluated on a weekly basis over the course of the flowering period. The numbers of bumblebees that were flying in and out were recorded every 10 min over the course of 90 min. In addition, the numbers of dead bumblebee workers present in each tent were recorded on a weekly basis.

Results

Determination of the concentration of *Beauveria bassiana* in the Prestop-Mix gave $4.5 \pm 0.1 \times$ CFU/g product.

Two-way ANOVA showed no significant ($P = 0.167$) interaction between the factors ‘treatment’ and ‘time’, and no significant ($P = 0.234$) effect of the factor ‘treatment’, whereas the factor ‘time’ resulted in a significant ($P = 0.001$) effect on the foraging activity. One week after the start of the greenhouse experiment, the foraging activities were 22.0 ± 3.5 for hive 1, 23.7 ± 1.5 for hive 2 and 26.7 ± 2.9 for hive 3. For week 2 the foraging activity in all treatments was also equal, showing no influence of the treatment. Moreover, the foraging activities for all nests in week 2 were not significantly different when compared with weeks 1 and 4 (paired *t*-test; $P = 0.644$ and $P = 0.698$ respectively).

The worker mortalities (i.e. the numbers of dead workers found outside the hive) were similar in T2, T3 and T4. The numbers of dead workers scored during weeks 2 to 4 were 20, 19 and 23 in treatments T2, T3 and T4 respectively. Thus, T3 (Prestop-Mix) and T4 (Prestop-Mix + Maizena-Plus) resulted in 0 – 15% worker mortality compared with T2 (Maizena-Plus alone).

Conclusion

Exposure to worker bumblebees to the biofungicide Prestop-Mix caused no adverse effects on bumblebee workers, with no loss of survival or impairment of flight activity of the workers during the 4 week flowering period.

MA 8.3/11

Citation: Ramanaidu, K & Cutler, GC (2013). Different toxic and hormetic responses of *Bombus impatiens* to *Beauveria bassiana*, *Bacillus subtilis* and spirotramat. *Pest Management Science*, (2013) 69(8): 949-954

Guideline: None

GLP: No

Summary

The effects of a biopesticide formulation of *Beauveria bassiana* on the common eastern bumblebee, *Bombus impatiens*, were evaluated. Microcolonies of bees were exposed to field-rate or lower concentrations, and data were collected over 60 days. Oral or topical application of *Beauveria bassiana* had no adverse effects on bees.

Materials and methods

Formulated *Beauveria bassiana* (Botanigard®ES; Koppert Canada Ltd, Scarborough, Ontario) was used in experiments. Serial dilutions were prepared in deionized water at concentrations of $2.33 \times$, $4.66 \times$, $1.16 \times$, $2.33 \times$ and $4.66 \times$ CFU/L. Controls consisted of treatment with water only.

Four 'class A' *Bombus impatiens* colonies were donated by Koppert Canada Ltd (Scarborough, Ontario). Two hives were used in contact exposure experiments, and two were used in oral exposure experiments. Each colony was provided with protein and sugar supplements from Koppert.

Experimental microcolonies were established in 461 mL plastic cups. The bottom of each cup was cut out and covered with netting (6.35 mm mesh) secured with a rubber band. Each cup was placed in a second 473 mL polystyrene container that contained a floral water pick or glass vial feeder stuffed with cotton wool or wicks soaked with a 60:40 honey:water solution. The feeder was placed under but touching the netting, allowing bees to feed on the honey solution *ad libitum*. Honey solutions and feeders were replaced twice per week.

Topical exposure

Four- to seven-day-old bees were used. For each treatment, 50 μ L of solution was applied to the dorsal thorax of each bee using a PAX 100-3 automatic microdispensing system. There were at least four replicate microcolonies, each containing three bees for each test concentration or control. As hives only produced 50–70 workers weekly, experiments were staggered approximately 1 week apart. After treatment, microcolonies were held at 25°C under total darkness and approximately 60% relative humidity. When separated from the queen of the parent commercial Koppert colony, one worker in each microcolony became dominant and soon thereafter began ovipositing. The other two workers assisted in brood rearing. As workers were unfertilized, all offspring produced were male drones.

Bees were checked once a day for 60 days. Data were collected on worker bee mortality, drone production, number of days to oviposition and number of days to drone emergence.

Oral exposure

Individual worker bees were selected and randomized among treatments, and microcolonies were established as described in the topical exposure section. *Beauveria bassiana* was administered orally to bees in 60:40 honey:water solution at $1.16 \times$, $2.33 \times$ and $4.66 \times$ CFU/L, offered in small glass vials stuffed with cotton. Bees in control microcolonies were given honey water only. Treatment solutions and feeders were replaced twice per week for 30 days. Bees were thereafter given untreated honey:water solution for an additional 30 days. There were five replicate microcolonies in each bioassay.

Data collection, endpoints measured and statistical analysis were conducted as in the topical exposure treatment, except that larval ejection data were also collected.

Findings

Topical exposure

There was no effect on *B. impatiens* worker survival when topically treated with *Beauveria bassiana* ($H=0.85$; $df=5$; $P=0.974$). Even at the highest concentration, a median of 3.0 workers remained after 60 days following treatment.

Beauveria bassiana topical treatments had no effect on drone production ($F=0.27$; $df=5, 18$; $P=0.922$), and there were no significant treatment – time interaction effects on drone production ($F = 1.05$; $df = 150, 540$; $P = 0.346$) (Table 8.3/10-1). Topical exposure to *Beauveria bassiana* also had no effect on days to oviposition ($F=0.94$; $df=5, 18$; $P=0.481$) or days to drone emergence ($F=1.65$; $df=5, 18$; $P = 0.204$).

Table 8.3/11-1. Effects on *Bombus impatiens* microcolonies 60 days after a topical treatment with *Beauveria bassiana*

Treatment concentration (CFU/L)	Mean (\pm 95% C.I.)		
	Drones produced	Days to oviposition	Days to drone emergence
Control	5.50 (5.88)	6.04 (3.16)	39.50 (2.33)
2.33 ×	5.00 (5.36)	6.00 (0.00)	38.67 (4.57)
4.66 ×	6.25 (3.76)	5.76 (2.32)	36.50 (1.62)
1.16 ×	6.25(7.84)	6.12 (4.24)	44.00 (10.21)
2.33 ×	5.25 (6.15)	4.74 (3.62)	33.67 (2.85)
4.66 ×	5.25 (5.26)	5.24 (4.14)	37.75 (2.58)

Oral exposure

There were no effects on worker *B. impatiens* survival after prolonged (30 days) feeding on diluted honey treated with *Beauveria bassiana* ($H = 4.93$; $df = 3$; $P = 0.177$).

Beauveria bassiana administered in honey water had no effect on drone production ($F = 0.82$; $df = 3, 16$; $P = 0.501$), and there was no treatment – time interaction ($F = 0.91$; $df = 90, 480$; $P = 0.715$) (Table 8.3/10-2). There was also no difference in the number of days to oviposition ($F = 0.62$; $df = 3, 16$; $P = 0.613$), number of larvae ejected ($F = 1.10$; $df = 3, 16$; $P = 0.377$) or days to drone emergence ($F = 0.31$; $df = 3, 16$; $P = 0.819$) with this treatment.

Table 8.3/11-2. Effects on *Bombus impatiens* microcolonies 60 days after an oral treatment with *Beauveria bassiana*

Treatment concentration (CFU/L)	Mean (\pm 95% C.I.)			
	Drones produced	Days to oviposition	Larval ejection	Days to drone emergence
Control	4.61 (6.31)	6.00 (0.62)	3.80 (3.47)	34.67 (1.73)
1.16 ×	7.82 (2.22)	5.40 (0.78)	3.20 (2.87)	33.00 (1.38)
2.33 ×	5.44 (4.78)	5.20 (1.57)	0.60 (1.18)	33.25 (2.31)
4.66 ×	6.43 (8.17)	6.00 (0.88)	1.80 (2.66)	34.25 (4.48)

Conclusion

Topical application of *Beauveria bassiana* at five concentrations ranging from of $2.33 \times$ to $4.66 \times$ CFU/L had no adverse effects on bees. Oral application of *Beauveria bassiana* at three concentrations ranging from of $1.16 \times$ to $4.66 \times$ CFU/L had no adverse effects on bees.

MA8.3/12

Citation: Mommaerts, V, Sterk, G, Hoffman, L & Smagghe, G (2009). A laboratory valuation to determine the compatibility of microbiological control agents with the pollinator *Bombus terrestris*. *Pest Management Science*, 65(9): 949-955

Guideline: None

GLP: No

Summary

A study was undertaken to identify any potential adverse side effects of the use of seven microbiological control agents (MCAs) on the bumblebee, *Bombus terrestris* L., in the context of combined use in integrated pest management. This summary will concentrate on effects of Botanigard® (*Beauveria bassiana* GHA). Bumblebee workers were exposed under laboratory conditions to each MCA at its maximum field recommended concentration (MFRC) via three different routes of exposure: dermal contact and orally via either treated sugar water or pollen.

Materials and Methods

All experiments were performed with worker bumblebees obtained from a continuous mass rearing programme and conducted under standardised laboratory conditions of 28 – 30°C, 60 – 65% RH and continuous darkness. The insects were provided *ad libitum* with commercial sugar water and pollen.

Newly emerged workers were collected from the bumblebee colony, and five were placed in artificial plastic nest boxes (15 × 15 × 10 cm). Four artificial nests were exposed for each treatment, and each experiment was repeated twice. Adult workers were exposed to the MCAs at their respective MFRCs via three different routes: via contact by topical application, and orally via treated sugar water and via treated pollen. For each treatment, worker mortality was scored after 72 h and on a weekly basis during a period of 11 weeks.

For the contact treatments, the treatments were prepared in water. Individual bees were topically treated with 50 µL of this aqueous solution on their dorsal thorax with a micropipette. For the oral treatments, bumblebee workers were continuously exposed to 500 mL of sugar water that was dosed with the MCA, or to pollen sprayed until saturation with the MCA in water. The treated sugar water and pollen were replaced weekly with freshly prepared material. Bees were treated with a concentration of $2.5 \times$ CFU/L.

Results

Lethal effects on worker survival

No lethal effects were exhibited against the workers during the first 72h following treatment, regardless of the different routes of exposure. However, after 11 weeks, worker mortality was recorded in nests that were treated with Botanigard®. $92 \pm 3\%$ of the workers were killed following topical treatment. In addition, when the concentration was reduced to 1/2, 1/5 and 1/10

of the MFRC, the mean worker mortality still reached $59 \pm 1\%$, $41 \pm 6\%$ and $46 \pm 4\%$, respectively, after 11 weeks. The presence of *Beauveria bassiana* GHA mycelium on the bodies of workers was also confirmed when cadavers were inspected under the microscope.

Sub-lethal effects on reproduction

The production of drones after 11 weeks was not significantly different ($P > 0.05$) from the control nests treated with water. Exposure of worker bumblebees via sugar water treated with Botanigard® for 11 weeks resulted in a reproductive rate that was not significantly different ($P > 0.05$) from that observed in the controls.

Pollen treatment with Botanigard® resulted in significant reduction in the production of males ($P < 0.05$). Although significant, this reduction of 16% is harmless according to the IOBC classification (Class 1).

Sublethal effects on behaviour

After 9 weeks exposure to Botanigard® worker mortality was 3%.

Conclusions

For bumble bees treated with Botanigard® at $2,5 \times$ CFU/L, severe worker mortality was observed after contact treatment of bumblebee workers, but not via the ingestion of treated sugar water or treated pollen where very low mortality occurred.

MA 8.4 Effects on Arthropods Other Than Bees

Table 8.4-1: Summary of non-target arthropod toxicity data

Species	Test type	MCPA	Endpoints	Reference/Source
Grassland arthropod community	Field	<i>Beauveria bassiana</i>	No reduction in numbers, diversity or species richness of soil dwelling invertebrate communities at spores/ha	Devotto <i>et al.</i> (2007)
			Significant difference between <i>Beauveria bassiana</i> at spores/ha and insecticide treatment up to 30 days after application	Devotto <i>et al.</i> (2007)
<i>Thanasimus formicarius</i>	Laboratory	<i>Beauveria bassiana</i>	>1 x spores/mL	Steinwender <i>et al.</i> (2010)

LIT = Literature review conducted in 2014

Beauveria bassiana strain PPRI 5339 is currently intended for glasshouse use only and no exposure to non-target arthropods is anticipated, as such no specific studies are necessary for a risk assessment. Non-target arthropod studies are underway with PPRI 5339, anticipating non-protected future uses and the reports will be available in 2015.

Summaries of the studies found in the literature conducted with *Beauveria bassiana* are presented below.

From Literature:

MA 8.4/01

Citation: Devotto, L, Cisternas, E, Gerding, M, Carrillo, R (2007). Response of grassland soil arthropod community to biological and conventional control of a native moth: using *Beauveria bassiana* and lambda-cyhalothrin for *Dalaca pallens* (Lepidoptera : Hepialidae) suppression. *Biocontrol*, 52(4): 507-531

Guideline: None

GLP: No

Summary

Two experiments were conducted in winter and spring in Chile to compare non-target effects of *Beauveria bassiana* (Balsamo) Vuillemin and lambda-cyhalothrin on the effects to non-target soil communities. This field study demonstrated that the application of *Beauveria bassiana* at a rate of spores/ha caused no reduction in numbers, diversity or species richness of soil dwelling invertebrate communities. The experiment was validated by including a 'toxic reference' treatment of λ-cyhalothrin for which an adverse impact on invertebrates would be expected. Indeed this was the case for this field evaluation.

Materials and methods

Experiments were set up in winter and spring. For the winter experiment three treatments were

prepared: control (0.5 ha); *Beauveria bassiana* (1 ha) and λ -cyhalothrin. Treatments were 100–150 m apart and surrounded by grassland. *Beauveria bassiana* was treated at the rate of spores/ha.

For the spring experiment, twelve plots (30 × 30 m) were established in a 4 ha field. Four plots were assigned to each treatment at random and four plots remained as control.

No additional plant protection products were applied. All treatments were applied using a horizontal bar mounted on a tractor.

Soil cores were collected from the spring experiment. A dilution plating method on agar-oat-dodine was used to enumerate the application rate and evaluate spore persistence. The persistence of spores on leaves was also evaluated.

The abundance and diversity of invertebrates were monitored by extracting soil cores three times in winter (before spraying and 20 and 40 days after spraying) and twice in spring (before spraying and 30 days after spraying). In the winter experiment, 120 cores were taken from every site, and 30 cores were taken from each plot in the spring experiment. In the laboratory, 70% of the cores were put on extraction trays and the remaining 30% were placed in Berlese-Tullgren funnels. Extraction was performed over 96 h. Specimens were examined under a stereoscopic microscope, counted and classified to the lowest possible taxonomic level.

Species richness curves, Shannon index, Hurlbert's PIE and dominance were evaluated. The foliage and soil counts of colony forming units (CFU) were analysed using a one way ANOVA.

Findings

The persistence of spores in the soil cores and on the leaves is presented in the Figure below:

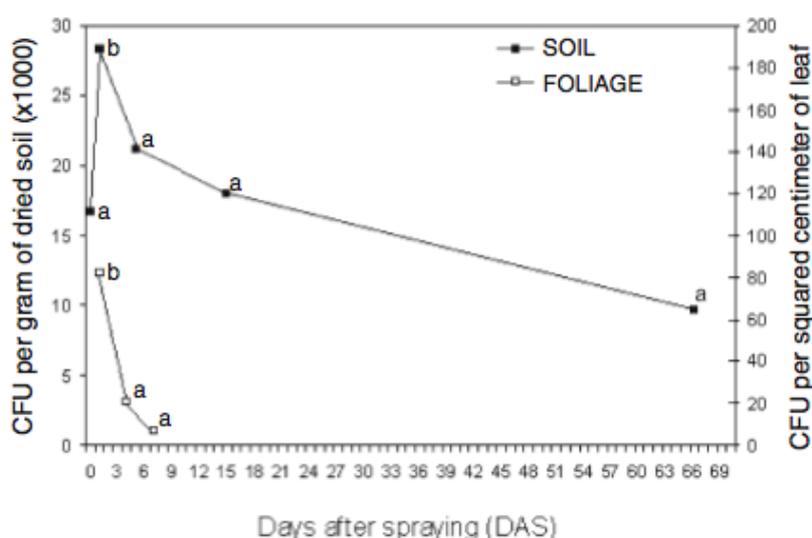


Fig. 8.4/01-1: Estimated numbers of *Beauveria bassiana* spores in soil and pasture foliage. On each curve, means followed by different letters differ according to Fisher's least significant difference test ($p < 0.05$).

The natural level of *Beauveria bassiana* spores in soil was 1.3 x CFU/g of dry soil (Fig. 1).

After spraying, the spore number increased by 70%, but they decline dramatically and the second post-spraying estimate did not differ from pre-treatment level ($p > 0.05$). In the foliage, spore number peaked 82 spores/ of leaf 1 day after spraying and the spore number by day 7 was close to zero.

A total of 9,555 invertebrates were identified in both experiments. Five taxa accounted for 84% of the total captures: Cantharidae (34%), Acari (30%), Curculionidae (9%), Carabidae (7%) and Araneae (4%). The functional groups represented were herbivores (50%), detritivores (34%), predators (14%) and omnivores (2%).

Diversity of invertebrate communities

In the winter experiment prior to treatment, diversity (estimated using Shannon index) at the control site was higher than both *Beauveria bassiana* and λ -cyhalothrin sites, while no significant differences were observed between these treated sites. Twenty days after a single spraying of *Beauveria bassiana* QU-B931 or λ -cyhalothrin, control and *Beauveria bassiana* sites showed no differences ($\alpha = 0.05$), while the diversity was significantly lower in the λ -cyhalothrin site. The same result was observed 40 days after spraying.

For the spring experiment, samples taken before spraying indicated that diversity in untreated and treated sites was similar. Thirty days after spraying, estimates of Shannon index showed no differences between control and *Beauveria bassiana* sites, while diversity at the λ -cyhalothrin site was significantly lower than both control and *Beauveria bassiana* sites.

Invertebrate community species richness

In the winter experiment, species richness estimated at $n = 495$ did not differ between sites before treatment, in spite of marked differences between site abundances (numbers at the *Beauveria bassiana* site were two-fold those at the control site). In both post-spraying sampling dates, species richness at the control site fell slightly out of the confidence interval calculated around the *Beauveria bassiana* rarefaction curve, while the λ -cyhalothrin rarefaction curve fell out of the lower confidence limit of the *Beauveria bassiana* rarefaction curve. Spring results indicated that the control and λ -cyhalothrin species richness were similar and higher than the *Beauveria bassiana* species richness before treatment, at $n = 402$. After treatment, the confidence limits of *Beauveria bassiana* and λ -cyhalothrin overlapped and included the control species richness, therefore the treatments did not alter species richness in this season.

Dominance

In the winter experiment, before treatment dominance was similar between sites assigned to *Beauveria bassiana* and λ -cyhalothrin, but it was lower at the control site before treatment applications. Twenty days after spraying, dominance remained high at the λ -cyhalothrin site (where the most dominant species accounted for more than 60% of catches), while dominant species represented just 45 and 39% of catches at the *Beauveria bassiana* and control sites, respectively. At 40 days after treatment, dominance at the control site was slightly higher than dominance at the *Beauveria bassiana* site, but lower than at the λ -cyhalothrin site. In the spring experiment, before spraying, no species accounted for more than 25% of catches (Figure 4, bottom left), but post-spray samples revealed that dominance was higher at the insecticide site (50%) than the dominance at the *Beauveria bassiana* (27%) and control sites (33%).

Conclusion

This field study demonstrated that the application of *Beauveria bassiana* at a rate of spores/ha caused no reduction in numbers, diversity or species richness of soil dwelling invertebrate communities. The experiment was validated by including a 'toxic reference' treatment of λ -cyhalothrin for which an adverse impact on invertebrates would be expected. Indeed this was the case for this field evaluation.

MA 8.4/02

Citation: Devotto, L, Cisternas, E, Carrillo, R, Gerding, M (2008). Non-target effects of *Dalaca pallens* Blanchard control examined through principal response curves: a guild approach in Southern Chile. *Chilean Journal of Agricultural Research*, (2008) 68 (3): 228-237.

Guideline: None

GLP: No

Summary

A field experiment was conducted to compare the effects of the biological control agent *Beauveria bassiana* (spores/ha) and the insecticide lambda-cyhalothrin (7.5 g active ingredient/ha) on non-target invertebrate species belonging to different guilds. The soil invertebrates were collected by using pitfall trapping and soil coring before and after spraying the biocontrol agent or the insecticide. Caught individuals were assigned to predator, herbivore or decomposer guilds and then a relatively new multivariate technique called principal response curves (PRC) was adopted to track the potential treatment effects over time in the abundance or activity of the non-target guilds. The products targeting *D. pallens* caused different effects on guilds: lambda-cyhalothrin decreased the activity-density of the predator guild but not herbivore or decomposer guilds, while the biological control agent caused no reduction in the activity-density of the three examined guilds. The negative effects of the insecticide were present from 1 to 60 days after treatment.

Materials and methods

The site was located in Chile in a naturalized pasture comprised primarily of *Lolium perenne* L. and *Holcus lanatus* L. A single application of *Beauveria bassiana* strain QU-B931 suspended in water, in a dose equivalent to spores/ha, or the pyrethroid lambda-cyhalothr at 7.5 g a.i./ha was sprayed onto 30 x 30 m open plots established in the pastures. A water treatment was included as a control.

Two techniques were used to sample the invertebrate fauna: pitfall trapping and Tullgren-Berlese funnel extractions of soil cores. Twelve traps were placed in the center of each plot for 4-day periods (one day before spraying and 1, 30, and 60 days after spraying). Thirty soil cores were extracted randomly from each plot, one day before and 30 days after spraying.

A completely randomized design with four replicates was used. Principal response curves (PRC) were used to analyze the time and treatment-dependent multivariate response of selected guilds. The method summarizes all the changes in activity-density of the species belonging to the same assemblage simultaneously, and the principal response, a weighted sum of the abundances of the taxa, is expressed as a canonical coefficient and reflects the behavior of the treated communities

relative to the untreated control.

Findings

Over 4111 specimens were identified. The most important taxa were the carabid beetles (42%), oribatid mites (20%), gnaphosid spiders (16%) and curculionid weevils (8%). The remaining taxa accounted for 14%. In terms of ecological functionality, 61% of the invertebrates were predators, 11% herbivores and 28% decomposers.

Fig 8.4/01 demonstrates the effects of the different treatments on the predator community. This demonstrates that the response of the communities treated with *Beauveria bassiana* do not differ from the untreated controls. This is not the case for the lambda-cyhalothrin. Also, Tables 8.4/01 and 8.4/02 show significant differences between the *Beauveria bassiana* and lambda-cyhalothrin treatments but not between the control and the lambda-cyhalothrin.

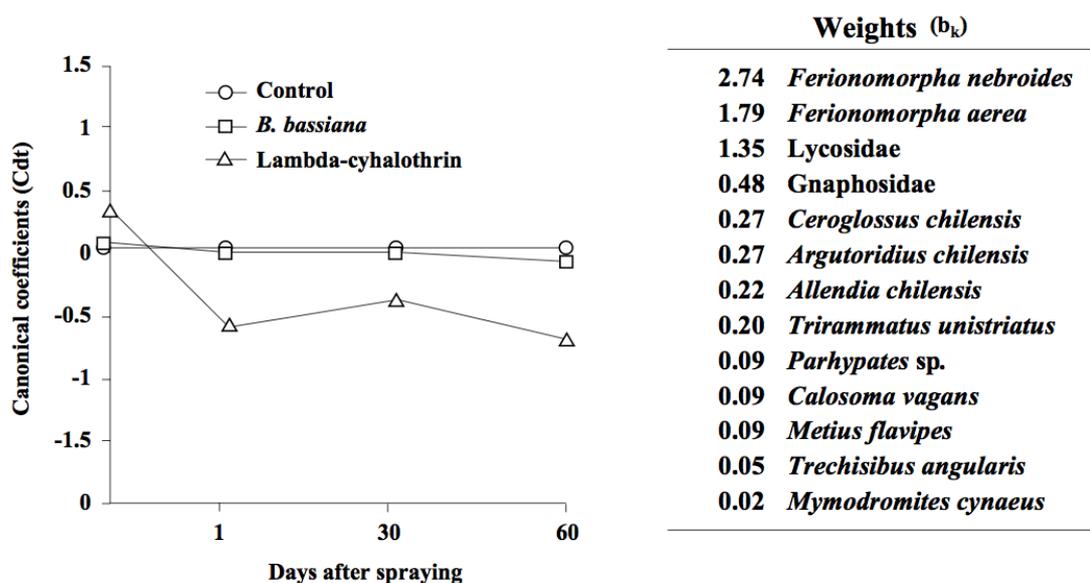


Figure 8.4/02. Principal response curve (PRC) for the predator guild from pitfall trapping, indicating the effects of a single spraying of *Beauveria bassiana* or lambda-cyhalothrin, compared to the control. Values deviating from the reference value of zero indicate treatment effects. Weights (right) indicate the affinity of the taxon to the PRC trend.

Table 8.4/02-1: Significance level of treatment effects in pitfall traps on the predators according to Monte-Carlo permutation tests (1999 permutations)

Comparisons	Before treatment	Days after treatment		
		1	30	60
P values				
Control vs. <i>Beauveria bassiana</i>	>0.16	>0.88	>0.62	>0.34
Control vs. lambda-cyhalothrin	>0.10	0.02	0.02	0.05
<i>Beauveria bassiana</i> vs. lambda-cyhalothrin	>0.76	0.02	0.02	0.10

Table 8.4/02-2: Significance level of treatment effects in the soil cores on the predator and non-target herbivore guilds 30 days after spraying according to Monte-Carlo permutation tests (1999 permutations)

Comparisons	Predators	Herbivores
	P values	
Control vs. <i>Beauveria bassiana</i>	0.77	0.79
Control vs. lambda-cyhalothrin	0.02	0.27
<i>Beauveria bassiana</i> vs. lambda-cyhalothrin	0.02	0.80

Conclusions

A field trial was conducted in Chile to compare the effects of *Beauveria bassiana* and Lambda-cyhalothrin on non-target arthropod communities. Lambda-cyhalothrin significantly reduced the predator guild compared to the *Beauveria bassiana* and control treatments for 30 days after treatments were applied. This clearly demonstrates that no unacceptable effects occurred on non-target arthropods when an application of spores *Beauveria bassiana*/ha were applied.

MA 8.4/03

Citation: Steinwender, BM, Krenn, HW, Wegensteiner, R (2010). Different effects of the insectpathogenic fungus *Beauveria bassiana* (Deuteromycota) on the bark beetle *Ips sexdentatus* (Coleoptera: Curculionidae) and on its predator *Thanasimus formicarius* (Coleoptera: Cleridae). *Journal of Plant Diseases and Protection*, 117(1): 33-38

Guideline: None

GLP: No

Summary

The effects of *Beauveria bassiana* on the bark beetle *Ips sexdentatus* (Coleoptera: Curculionidae) and its predator *Thanasimus formicarius* (Coleoptera: Cleridae) were tested. *T. formicarius* was inoculated with varying levels of severity. The for exposure of adult *T. formicarius* to *Beauveria bassiana* was >1 x spores/mL.

Materials and methods

Three treatment regimes were used. One of these was termed ‘normal’ and involved 30 beetles being treated with dry conidia of *Beauveria bassiana* on the last distal sternites. The other treatment, which was considered to be ‘extreme’ involved covering the whole ventral side of the body of 15 beetles with dry conidia. The concentration of colony forming units in the dry conidia was not cited. The third treatment involved dipping the beetles for 3 seconds in a conidia suspension which had a concentration of 1 × spores/mL.

The beetles were maintained individually at 20 ± 1°C and were fed with one untreated *Ips sexdantus* per day.

Findings

Mortality and feeding assessments of the treated beetles are given in the table below.

Table 8.4/03-1: Mean life span (MLS), mortality and feeding behaviour of *Thanasimus formicarius* after inoculation by *Beauveria bassiana*

Treatment	No. of beetles	MLS (days) [S.D.] ^c	No. of dead beetles	Mean <i>I. typographus</i> consumed/day [S.D.] ^c
DC 'extreme'	15	52 [± 24] ^a	7	0.56 [± 0.14] ^a
DC 'normal'	30	151 [± 68] ^b	0	0.61 [± 0.17] ^a
1 x conidiospores/mL	30	157 [± 85] ^b	0	0.62 [± 0.11] ^a
Control (untreated)	30	188 [± 78] ^b	0	0.73 [± 0.15] ^a

^c Values with different letters represent significant difference ($P < 0.05$)

The results demonstrate that only the 'extreme' dry conidia treatment resulted in significant adverse effects on adult *T. formicarius*. As no information was provided on conidia concentrations in this treatment it is assumed that the *Beauveria bassiana* was applied at $>1 \times$ spores/mL.

Conclusions

The for exposure of adult *T. formicarius* to *Beauveria bassiana* was $>1 \times$ spores/mL.

According to Zimmermann:

It is well known that *Beauveria bassiana* has a wide host range, occurring on several hundred arthropod species; however, host specificity is really a strain-specific trait. For example, *Beauveria 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) (Doc M-MA 8.4/04). The GHA strain of *Beauveria 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.

Generally, there is a difference between the physiological host range and the ecological host range (Hajek & Butler 2000) (Ref M-MA 8.4/05). 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) (Doc M-MA 8.4/06) and Jaronski *et al.* (2003) (Doc M-MA 8.4/07).

One of the first comprehensive reports was given by Goettel *et al.* (1990), who listed the effects of *Beauveria bassiana* on nontarget invertebrates, such as bees and other pollinators, silkworms, predators and parasitoids. Further, general information is mentioned by Goettel *et al.* (1990, 2001) (Doc M-MA 8.4/08, Ref M-MA 8.4/09) and Vestergaard *et al.* (2003) (Ref M-MA 8.4/10). The last authors conclude that despite the wide host range of *Beauveria bassiana*, evidence to date suggests that

this fungus can be used with minimal impact on non-target 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 under stress conditions, while honey bees and earthworms were not affected. 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) (Ref M-MA 8.4/10).

MA 8.5 Effects on Earthworms

In an OECD workshop² the relevance of earthworm tests was discussed. Adverse effects have never been observed in earthworm tests with microbial control agents, this probably being due to the highly developed immune system of the organisms. It was decided that earthworm tests are not required unless the microbial is not naturally occurring in the soil. Given that *Beauveria bassiana* naturally occurs in the soil worm tests are not required.

Effects on other soil invertebrates

From Zimmermann (2007):

Beauveria 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.

Beauveria bassiana was also found in high frequency on a great number of the collembolan *Onychiurus subtenuis* (Visser *et al.* 1987) (Doc M-MA 8.5/01). The authors concluded that there was no indication that *Beauveria bassiana* killed the collembolan. The collembolan *Folsomia candida* was not susceptible to *Beauveria bassiana*. It consumed and inactivated the insect pathogen without causing mortality or any other harmful effects (Broza *et al.* 2001) (Doc M-MA 8.5/02). Pathogenicity tests of *Beauveria bassiana* and *B. brongniartii* were conducted against adults of *Folsomia fimetaria*, *Hypogastrura assimilis* and *Proisotoma minuta* (Dromph & Vestergaard 2002) (Doc M-MA 8.5/03). By dipping the collembolans in 1 x conidia/mL suspension and in one case also in 1 x conidia/mL, 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 x conidia/g 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 (Samšišákova & Samšišák 1970; Dromph 2001, 2003) (Doc M-MA 8.5/04, Doc M-MA 8.5/05, Doc M-MA 8.5/06). For example, *Beauveria bassiana* is distributed both in a horizontal and vertical direction by the mite *Sancassania phyllognathi* which is resistant to fungal infection (Samšišákova & Samšišák 1970). The transmission of spores of *Beauveria 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) (Doc M-MA 8.5/06).

These findings show that there are no or very low detrimental effects on the tested soil-dwelling

² OECD/KEMI/EU Workshop on Microbial Pesticides. 17 – 19 June (2013), Saltsjöbaden, Sweden. OECD Biopesticides Steering Group & OECD Task Force on Biocides.

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.

MA 8.6 Effects on Non-Target Soil Micro-Organisms

Table 10.6-1: Summary of soil micro-organisms toxicity data

Species	Test type	MCPA	Endpoints	Reference/Source
Soil micro-organisms	Field study	<i>Beauveria bassiana</i>	No adverse effects at 3 × conidia/	Hirsch <i>et al.</i> (2013)/LIT

LIT = Literature review conducted in 2014

From Zimmermann (2007):

Investigations on the natural prevalence of *Beauveria 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) (Doc M-MA 8.6/01). 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) (Doc M-MA 8.6/02). Similar results were also obtained by Wang *et al.* (2004) (Doc M-MA 8.6/03) in *Beauveria bassiana* and Enkerli *et al.* (2004) (Doc M-MA 8.6/04) in *B. brongniartii*. Wang *et al.* (2004) monitored the fate of inundatively applied strains of *Beauveria 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 *Beauveria 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) (Doc M-MA 8.6/05).

There are several reports on interactions of *Beauveria* spp. with hyperparasitic, antagonistic and especially, phytopathogenic fungi. A hyperparasitic fungus attacking *Beauveria bassiana* and *B. brongniartii* is the ascomycete *Syspastospora parasitica*, formerly known as *Melanospora parasitica* (Markova 1991; Posada *et al.* 2004). Lingg and Donaldson (1981) (Doc M-MA 8.6/06, Doc M-MA 8.6/07, Doc M-MA 8.6/08) reported that the survival of conidia of *Beauveria 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 *Beauveria bassiana*. There are also various interactions with *Clonostachys* spp. and *Trichoderma* spp. which may suppress or overgrow *Beauveria bassiana* *in vitro* (Krauss *et al.* 2004; Zimmermann unpublished) (Doc M-MA 8.6/09). There has been increased interest to test and use *Beauveria bassiana* also against plant pathogens (Ownley *et al.* 2004) (Doc M-MA 8.6/10). According to the experiments, *Beauveria 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) (Doc M-MA 8.6/11) had previously observed that *Beauveria 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) (Doc M-MA 8.6/12)..

From literature:

MA 8.6/13

Report: Hirsch, J, Galidevara, S, Strohmeier, S, Devi, KU, Reineke, A (2013). Effects on Diversity of Soil Fungal Community and Fate of an Artificially Applied *Beauveria bassiana* Strain Assessed Through 454 Pyrosequencing. *Microbial Ecology*, 66(3): 608-620

Guidelines: None

GLP: No

Summary

A case study was conducted using tag- encoded 454 pyrosequencing of fungal ITS sequences to assess the fate and potential effect of an artificially applied *Beauveria bassiana* strain on the diversity of soil fungal communities in an agricultural field in India. Overall fungal diversity was not influenced by application of *Beauveria bassiana* during the 7 weeks of investigation.

Materials and methods

Experiments were conducted from October to December in a cultivated agricultural with a standing crop of 4-week-old chili plants. Chili was chosen as a crop in this study, as it is frequently attacked by insect pests such as *Spodoptera litura* and *Helicoverpa armigera* (Lepidoptera: Noctuidae) that are, at the same time, potential targets for the applied *Beauveria bassiana* isolate. No naturally occurring entomopathogenic fungal epizootics have been documented in the selected field over the last 15 years and the last artificial introduction of *Beauveria* sp. was made 14 years previously. In addition, no insecticides or fungicides had been applied in the field over the previous 2 years. Two 50 plots were prepared. One of them was treated (T) once with *Beauveria bassiana* strain ITCC 4688 and the other was left as an untreated control (C). There was no replication. Before the application of *Beauveria bassiana*, soil cores (approximately 4 × 4 × 15 cm depth) were collected in order to determine pre-treatment levels of *Beauveria bassiana*.

For application of the fungus to the treated (T) plot, 200 g of rice containing sporulated

Beauveria bassiana strain ITCC 4688 was suspended in 30 L water with 2 mL Tween 80 to give a final concentration of ~ conidia per mL. The whole suspension was dispensed manually onto the soil and plants in the (T) plot, resulting in a concentration of approximately $3 \times$ conidia/50 .

For assessment of effects of this *Beauveria bassiana* strain on indigenous soil fungal community structure, soil samples were collected in each plot as described above at weekly intervals for a duration of 7 weeks.

In the laboratory the soil samples were homogenized and genomic DNA was extracted. In total, 92 fungal PCR products, tag- encoded according to sampling date and plot, were pooled at equimolar concentrations and 454 pyrosequencing was performed commercially on a Roche (454) FLX Genome Sequencer.

Findings

Prior to the application of *Beauveria bassiana*, no significant differences in the diversity (Shannon index) of fungal communities between control and treatment plots were evident.

After application, a remarkable increase in the number of sequence reads homologous to *Beauveria bassiana* and *C. bassiana* were observed in samples from both the control and treatment plots, with the highest number of *Beauveria bassiana*.

During the 7 week investigation, no effect of *Beauveria bassiana*, applied at a concentration of $3 \times$ conidia per 50 , was evident on the diversity of indigenous fungal communities.

Conclusion

At an application rate of *Beauveria bassiana* at $3 \times$ conidia/ no adverse effect on indigenous fungal communities was observed.

MA 8.7 Additional Studies

Effects of *Beauveria bassiana* on additional taxa to those covered in previous sections were also described by Zimmermann (2007) as presented below:

Amphibia

A fungal suspension of *Beauveria bassiana* was fed to the leopard frog, *Rana pipiens* via gastric incubation. The dosage was $9.8 \times$ conidia. 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) (Doc M-MA 8.7/01).

Reptiles

A fungus attributed to be *Beauveria bassiana* was observed to cause infections in a captive American alligator (Fromtling *et al.* 1979) (Doc M-MA 8.7/02). 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 spores of *Beauveria bassiana* into the lung, no mortality was observed,

while a second contaminated tortoise died when kept only at 16°C (Müller-Kögler 1967) (Doc M-MA 8.7/03).

Non-target plants

In summarising the past literature, Müller-Kögler (1967) concluded that side-effects or any phytopathogenic activity on plants are not known. *Beauveria bassiana* has been reported to be an endophyte of certain plants, especially corn (Bing & Lewis 1992) (Doc M-MA 8.7/04). Studies have demonstrated that the fungus, applied to whorl-stage corn by foliar application or injection, colonised, translocated and persisted in corn plants. In the greenhouse, *Beauveria bassiana* was applied as a liquid seed treatment to Bt transgenic corn hybrids and their near isolines (2 x conidia/mL), and no significant differences in seed germination or presence of root pathogens were observed (Lewis *et al.* 2001) (Doc M-MA 8.7/05).

***Beauveria bassiana*
strain PPRI 5339**

DOCUMENT M-MA, Section 9

**SUMMARY AND EVALUATION OF
ENVIRONMENTAL IMPACT**

Version

Date	Data points containing amendments or additions and brief description	Document identifier and version number

¹ It is suggested that applicants adopt a similar approach to showing revisions and version history as outlined in SANCO/10180/2013 Chapter 4 How to revise an Assessment Report

Table of Contents

MA 9	SUMMARY AND EVALUATION OF ENVIRONMENTAL IMPACT.....	4
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MA 9 SUMMARY AND EVALUATION OF ENVIRONMENTAL IMPACT

Please refer to MP 11 for a summary and evaluation of environmental impact.

Beauveria bassiana
strain PPRI 5339

DOCUMENT M-MA, Section 10

CLASSIFICATION AND LABELLING OF THE
ACTIVE SUBSTANCE

Version

Date	Data points containing amendments or additions and brief description	Document identifier and version number

¹ It is suggested that applicants adopt a similar approach to showing revisions and version history as outlined in SANCO/10180/2013 Chapter 4 How to revise an Assessment Report

Table of Contents

MA 10	CLASSIFICATION AND LABELLING OF THE ACTIVE SUBSTANCE	4
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MA 10 CLASSIFICATION AND LABELLING OF THE ACTIVE SUBSTANCE

The active substance *Beauveria bassiana* strain PPRI 5339 is a microorganism. Therefore no classification and labelling scheme is required as Regulation (EC) No 1272/2008 (CLP) is only applicable to substances, mixtures and articles.

In M-MA Section 5, the metabolite beauvericin is considered to be genotoxic, based on positive *in vitro* screening tests. However, analytical data on the active substance (see M-MA 1.4.3) indicates that no beauvericin was observed in the active substance above the limit of detection of the analytical method of 0.1 mg/kg (w/w). On the assumption that the active substance may contain beauvericin at a concentration up to 0.1 mg/kg, this would not trigger a genotoxic classification as it is well below the concentration limits associated with genotoxic classifications defined in the CLP Regulation.

In conclusion, no formal classification requirements, including pictograms, signal words, hazard statements or precautionary statements are required for the active substance.

The only phrase that must appear on the label for the active substance relates to the assumption that all microorganisms are considered to be potential allergens and is worded as follows:

“May invoke a sensitising reaction”.