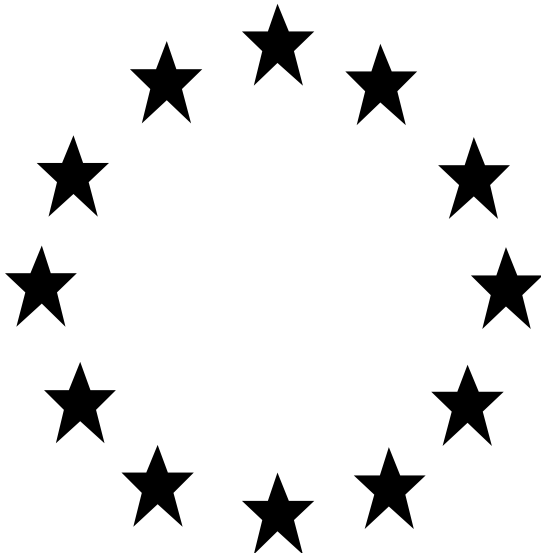

Draft Assessment Report (DAR)

- public version -

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

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Annex B

Beauveria bassiana GHA

B-6: Toxicity, pathogenicity and infectivity

B.6 Effects on human health

B.6.1 Tier I - The active micro-organism

B.6.1.1 Basic information (OECD IIM 5.1)

GHA is one of many strains of the entomopathogenic fungus *Beauveria bassiana*, a cosmopolitan and ubiquitous soil saprophyte. *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 micro-organism 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 *Beauveria bassiana* strains do not survive and replicate at temperatures higher than 35 ^{o}C but this, of course, does not exclude the possiblity of at least local effects that may be caused by a micro-organism just after it has entered the host body. More convincing, specific studies for infectivity, pathogenicity and toxicity have been performed with strain GHA in laboratory animals and are reported under B.6.1.2 and B.6.2. 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 intratracheal application that might be partly mediated by an immune response of the mammalian host. This observation seems to be in line with the inhalation allergy occuring in humans. In this section, available experience with *B. bassiana* in humans is summarised and evaluated.

B.6.1.1.1 Medical data

No epidemiological studies on the presence of *Beauveria 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, few anecdotal reports from the open literature on the isolation of *Beauveria 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.

B.6.1.1.2 Medical surveillance on manufacturing plant personnel

The notifier claimed that no infectivity, pathogenicity, toxicity or sensitisation effects caused by *Beauveria bassiana* strain GHA were reported from the manufacturing site in Butte (Montana, U.S.A.). Results of an occupational health survey in this plant for the years 2000 - 2004 (Chatriand, 2001-2005; TOX2006-901) have been submitted confirming the lack of health effects in employees (total number not given) who had been in contact with that micro-organism.

B.6.1.1.3 Sensitisation/allergenicity observations, if appropriate

Westwood *et al.* (2005, TOX2007-482) used crude extracts of *Beauveria 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 *Beauveria bassiana*-specific proteins that might cause allergies as well as

indication for cross-sensitisation because of certain reactive epitopes that this micro-organism 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 *Beauveria 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 reactions because of sensitisation and irritation, it must be seriously taken into account that four of five people who had reported previous occupational exposure to *Beauveria 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.* (1985, TOX2007-470) examined patients with recurrent allergic bronchial obstruction and identified *Beauveria 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 *Beauveria bassiana* spores in air samples was rather low.

Henke *et al.* (2002, TOX2006-2716) reported allergic alveolitis in an immunocompromised patient suffering from disseminated infection with *Beauveria spp.* (see below).

Semalulu *et al.* (1992, TOX2007-480) cited references (not available to the RMS) that claimed occurrence of moderate or even severe allergenic reactions among scientists working with *Beauveria bassiana* but also mentioned papers suggesting that there were no deleterious effects in workers handling this fungus.

Based on all this information from the literature, and taking into consideration the observations in laboratory animals (for details see section B.6.1.2), there is enough evidence to consider *Beauveria bassiana*, irrespective of the strain, a potential human allergen by both skin and inhalatory contact requiring classification and labelling.

B.6.1.1.4 Direct observation, e. g. clinical cases

Few cases are reported in the open literature that *Beauveria bassiana* (strain never specified) was isolated from people who suffered from various diseases.

Eye infections

Low *et al.* (1997, TOX2007-477) described the case of a healthy 67 years old woman in Australia who experienced *Beauveria 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 synchiae 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 month 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 Sabouraud's agar where anterior chamber and corneal specimens had been plated and puff-balls became visible in 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 *Beauveria bassiana*.

Kisla *et al.* (2000, TOX2007-475) 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 infammatory 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 *Beauveria 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, ASB2007-4608) reported infection and eventually penetration of the cornea by *Beauveria 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 couse of an eye infection with *Beauveria bassiana* was described by Ishibashi et al. (1984, ASB2007-4606).

In summary, these ophthalmological case reports do not point to a special risk of *Beauveria bassiana* strain GHA to cause eye infections in operators since adverse effects were only seen after massive mechanical damage to the cornea that allowed invasion of the fungus into the

eye. There are no reports on keratitis or other ocular disease in humans with intact cornea. However, these findings suggest a possible affinity of *Beauveria bassiana* to ocular tissues when the cornea once had been penetrated. This was partly confirmed by Ishibashi *et al.* (1987, TOX2007-474) who reported local corneal infection after experimental ocular infection of rabbits (for details see section B.6.2). Richardson et al. (1993, ASB2007-4607) have shown that *Beauveria bassiana* may contaminate and grow in soft contact lenses. However, no cases of infection from that source have been reported so far.

Systemic disease

Henke *et al.* (2002, TOX2006-2716) 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. After plating, fungal colonies were observed resembling either *Beauveria bassiana* or the related species *Beauveria brongniartii* but genotyping (sequencing) pointed rather to the latter one. The woman 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 leucemia was reported by Tucker *et al*. (2004, TOX2006-2723). 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.

Isolated cases of lung infections in humans by *Beauveria bassiana* were reported long ago already by Freour *et al.* (1965) and Kuru (1932). The original papers were not available to the RMS but were referred to by Ishibashi *et al.* (1987, TOX2007-474) and in other sources. Of course, it cannot be expected that the methods of species identification at that time complied to modern standards.

Gürcan *et al.* (2006, TOX2007-473) 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 *Beauveria bassiana*

Lackner *et al.* (2004, TOX2007-476) 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 ubiquitious occurrence of *Beauveria bassiana*.

In South Korea, silk moth (*Bombyx mori*) larvae infected with *Beauveria bassiana* are currently in use in traditional medicine as drugs for the therapy of stroke and stroke-induced speech problems, headache, tremor, convulsions, tonsillitis or pharyngitis, urticaria, lymphedema, mastitis, rubella, or tubercular lymphadenitis (Pemberton, 1999, TOX2007-479).

B.6.1.2 Basic studies (OECD IIM 5.3)

Table B.6.1-1 summarises the studies with *Beauveria bassiana* strain GHA in laboratory animals which were submitted by the notifier to prove that this micro-organism was neither infective, pathogenic nor toxic.

Table B.6.1-1: Summary of the acute studies

Annex II point	Study/Route/ Method	Species	Dose per animal	Results	Conclusion	Reference
IIM 5.3.1	Skin sensitisation (Buehler test, 3 inductions)	Guinea pig	80 mg (*approx.* 8×10^9 CFU)	Negative (not sensitising)	No final conclusion can be drawn	Findlay (1998, TOX2006-870)
IIM 5.3.2	Oral gavage	Rat	*approx.* 1×10^8 CFU	No evidence of toxicity, pathogenicity or infectivity; rapid clearance (by day 3 post dosing)	$LD_{50} > 1 \times 10^8$ CFU; not to be classified, no concern	Barbera (1993, TOX2006-871)
IIM 5.3.3	Intratracheal	Rat	*approx.* 1×10^8 CFU	No evidence of infectivity but local effects on the lungs (inflammation/immune reaction, organ weight increase), transient reduction in body weight gain; clearance complete by day 7 post dosing	$LC_{50} > 1 \times 10^8$ CFU; evidence of some inhalation toxicity, sensitisation by inhalation cannot be excluded	Barbera (1993, TOX2006-872)
IIM 5.3.4	Intraperitoneal	Rat	*approx.* 1×10^7 CFU	No evidence of toxicity, pathogenicity or infectivity; rapid clearance (by day 3 post dosing)	$LD_{50} > 1 \times 10^7$ CFU; not to be classified, no concern	Barbera (1993, TOX2006-873)
IIM 5.5.1	Dermal	Rabbit	1.6×10^{11} CFU	No systemic effects but signs of slight but persisting local irritation	$LD_{50} > 1.6 \times 10^{11}$ CFU; not to be classified, no concern	Johnson (1993, TOX2006-874)

Thus, a basic set of valid acute studies on *Beauveria bassiana* strain GHA has been performed using the oral, intratracheal and intraperitoneal routes. In addition, a dermal sensitisation study according to the method of Buehler is available. An acute dermal study is usually not required for micro-organisms but was submitted for this fungus, too. This study (Johnson, 1993,

TOX2006-874) is reported in detail under section B.6.2 but is mentioned in the summary table to give a more comprehensive picture on the possible infective, pathogenic and toxic properties of this micro-organism.
In the following, the original studies submitted by the notifier are reported in detail.

B.6.1.2.1 Sensitisation

Reference: IIM 5.3.1/01

Report: Findlay, J. (1998): Dermal sensitisation study of *Beauveria bassiana* strain GHA in Guinea pigs using the Buehler method. IIT Research Institute, Chicago, U.S.A. on behalf of Mycotech Corp. Unpublished Report No:L08608 SN30.
TOX2006-870

Guidelines: OECD Guideline 406 (According to the notifier, US EPA 40 CFR Part 158.740 and OPPTS 870.2600 were also followed but this was not checked by the RMS).

Deviation (from OECD 206): None

GLP: Yes (self-certified in accordance with US 40 CFR 160).

General assessment: The study is considered supplementary since a Buehler assay with only 3 inductions is generally considered not sufficient to exclude a skin sensitising potential. Furthermore, the relevance of this test for checking the potential allergenicity of micro-organisms is doubtful.

Material and methods:

The sensitising potential of *Beauveria bassiana* strain GHA was studied in guinea pigs using the Buehler method. The treated group consisted of 20 male guinea pigs (Hartley strain from Charles River Laboratories, 3 - 4 weeks old). Food and drinks were administered *ad libitum* and animals were housed individually in suspended stainless cages between 22 and 27 °C and at relative humidity between 31 and 76 %. A 12 hour light/dark cycle was maintained throughout the study.

The test substance (Lot no. 98-07-1 containing about 1×10^{11} conidia per gram with a spore viability of 95 %) was applied undiluted at an amount of 80 mg (approximately 8×10^{9} conidia) by means of a Hill Top Chamber to the shaved backs of 20 male guinea pigs during an induction phase of 3 weeks (*i.e.* 3 successive treatments at weekly intervals).
The negative control group was 10 male guinea pigs, handled in the same manner but not treated with the fungus. The positive control group of 10 male guinea pigs was treated with undiluted hexylcinnamaldehyde (HCA) which is a known sensitising agent.

Two weeks after the last induction, the animals in the *Beauveria bassiana*-treated and negative groups each received an epicutaneous challenge dose of the test substance, and the positive group received a dose of 15 % HCA in corn oil. All animals were observed for

erythema or other dermal reactions approximately 24 or 48 hours after removing of the wrappings following the first induction and the challenge dose.

Findings:

There were no mortalities in the study.
No positive reactions (*i.e.*, an erythema score of > 1) were observed in the treated or negative control groups following the induction and challenge doses. In the positive control group treated with HCA, 40 % exhibited a positive response, thus proving the validity of the study.

Table B.6.1-2: Buehler test with *Beauveria bassiana* strain GHA in guinea pigs - Responses to challenge applications

Group	No. of animals	Incidence of significant responses		Total responders
		24 hours	48 hours	
Control negative	10	0	0	0
Control positive (HCA)	10	4	4	4
Test	20	0	0	0

Note: negative control animals were not treated during the induction phase.

Conclusion:

Beauveria bassiana strain GHA was not a skin sensitiser to guinea pigs under the conditions of this study. Based on the negative outcome of the Buehler test, the notifier expressed its opinion that no classification for dermal sensitisation was warranted. However, according to the evaluation practice for pesticides in the EU, the Buehler test in general is considered inferior to the more rigorous Magnusson-Kligman maximisation test with regard to reliability and predictivity. In particular a Buehler test with only 3 inductions is usually not accepted as a convincing proof for the lack of a skin sensitising potential. For micro-organisms in general, it appears doubtful whether an assay without intradermal induction in fact can produce sensitisation because the bareer function of intact skin has evolved to avoid the entry of potentially pathogenic germs as one of its main tasks. Thus, a meaningful conclusion on skin sensitisation by strain GHA cannot be drawn from this study.
The formulation BotaniGard 22 W proved positive in a Buehler test with three inductions (Arcelin, 2006, 1679375, see section B.6.4.1). According to the considerations above, this is rather assumed to be due to co-formulants than to the micro-organism itself. Classification and labelling of the plant protection product is needed in any case.

B.6.1.2.2 Acute toxicity, pathogenicity and infectiveness

Acute effects of strain GHA were investigated in rats using the oral, intratracheal and intraperitoneal routes. *Beauveria bassiana* is known to produce a wide variety of toxic compounds (Fuguet and Vey, 2004, TOX2007-483). In fact, the three *Beauveria bassiana* secondary metabolites of biggest concern, *i.e.*, bassianolide, beauvericin, and oosporein, have

usually not been detected in liquid cultures of strain GHA or in the plant protection product (Bradley, 1993, TOX2006-898). As reported in Volume 4 of this DAR, the amount of beauvericin and bassianolide was in most batches below the detection limit of 5 ppm. In the single batch (lot) 930210 that was used in the single dose studies for infectivity, pathogenicity and toxicity, however, higher concentrations of beauvericin (48 ppm) and bassianolide (51 ppm) were detected. Thus, possible toxic effects of these two metabolites were at least partly covered by these studies.

B.6.1.2.2.1 Acute oral toxicity, pathogenicity and infectiveness

Reference: IIM 5.3.2/01

Report: Barbera, P. W. (1993): Toxicity/pathogenicity testing of *Beauveria bassiana* strain GHA following acute oral challenge in rats. IIT Research Institute, Chicago, U.S.A. on behalf of Mycotech Corp. Unpublished Report No. L08433, Study no. 6.
TOX2006-871

Guidelines: Currently, there is no OECD or EU guideline for studies of this type with micro-organisms. It is stated in the report that U.S. EPA 152A-10 was followed which is comparable to OPPTS Guideline 885.3050.

Deviations: None

GLP: Yes (self-certified in accordance with US 40 CFR 160)

General assessment: The study is considered acceptable.

Material and methods:

Two groups of 9 male and 9 female fasted CD rats (supplier: Charles River Labs., Kingston/New York, U.S.A.) were given a single oral dose of conidia (spores) of *Beauveria bassiana* strain GHA (Lot No. 930210GHA-A) by gavage at a nominal dose (target concentration) of about 1×10^8 CFU per animal. It is stated in the report that the concentration was chosen on the basis of EPA recommendations for testing of micro-organisms. The first dose group (TS) received the viable micro-organism at actual concentrations of 1.05×10^8 CFU in male animals and 1.03×10^8 CFU in females. A second dose groups of the same size and composition (KTS) was administered killed (autoclaved for 20 minutes at 121 ºC) *Beauveria bassiana* strain GHA spores at rates of 1.08×10^8 CFU (males) and 1.05×10^8 CFU (females). Taking into account the mean body weight of male and female rats on the day of dosing and an amount of 8×10^{10} CFU per gram test substance, this would correspond to a dose of 5.7 mg technical as/kg bw in males and of 6.6 mg technical as/kg bw in females.
Two separate control groups were included, *i.e.*, a naïve control (9 rats per sex) and a shelf control of 3 males and 3 females only. The latter group was intended to check for horizontal transfer of the micro-organism and was kept in the same animal room as the TS group. It is not clearly stated in the report if the control animals received the dilution medium by oral gavage or remained completely untreated.

Food and fresh water were administered *ad libitum* and animals were housed individually in suspended polypropylene cages at a temperature between 23 – 27 ºC and a relative humidity between 39 and 66 %. A 12 hour light/dark cycle was maintained throughout the study.
Following adaptation to the laboratory conditions, the rats were fasted for 18 - 22 hours and dosed. To prepare the dosing solutions, the test substance (provided in the physical state of a white powder with 14.3 % conidia content as certified by the sponsor) was diluted in purified water (100 mg in 118 mL) containing 0.1 % Tween 80 and mixed by vortex for 1 minute.

The animals were monitored daily for clinical signs of toxicity or pathogenicity. Body weight was determined prior to randomisation, at the time of application (day 0) and on days 3 and 7. Three rats per group and sex were sacrificed at days 0, 3 and 7 post dosing by carbon dioxide asphyxiation with the exception of the shelf control animals that were all killed at study termination after 7 days. The study was finished on day 7 already (instead of the usual 14-day post-observation period) because of the lack of clinical symptoms and because no conidia could be detected in the rats that were sacrificed and examined on day 3. All animals were subjected to gross necropsy with special attention to the following organs and tissues: blood, brain, heart, intestinal tract (caecum, small intestine, stomach), kidneys, liver, lungs and associated lymph nodes, mesenteric lymph nodes, and spleen. Organ weights were determined for brain, caecum, kidneys, liver, lungs and associated lymph nodes, mesenteric lymph nodes, spleen and stomach but in the summary tables only relative values are given although individual absolute weights are available. Histology was not performed and tissues were not saved.

As part of this study, microbial clearance was investigated. Previously, the sensitivity of the detection method was determined on both selective (Dodine oatmeal agar, DOA) and non-selective fungal recovery media (Sabouraud dextrose agar with yeast extract, SDAY, and potato dextrose agar) using fungus-inoculated lung and caecal samples prior to and post homogenisation. For microbial enumeration in the main study, the following organs and tissues were processed: blood, brain, caecum, kidneys, liver, lungs and associated lymph nodes, mesenteric lymph nodes, spleen and stomach. Following homogenisation, samples were plated on either DOA or SDAY and incubated for at least 5 days at room temperature before colonies were counted. All analytical determinations were conducted in duplicate or triplicate and plates were checked for contamination.

Findings:

There were no mortalities in any group in the study and no clinical signs of toxicity or pathogenicity were observed. Body weight (gain) was not altered and there was no evidence of an impact of the micro-organism on organ weights. Necropsy did not reveal remarkable pathological changes.

Conidia were detected soon after dosing (day 0) in stomach and caecum in both sexes and, in addition, in the spleen of one female rat but these observations were confined to the TS group. By day 3, all traces of *Beauveria bassiana* had cleared from the body indicating a lack of colonisation and infectivity. It is interesting to mention that no conidia were found in blood, even not on day 0. *B. bassiana* was not detected in any other dose group (including the KTS group receiving non-viable spores) at any time. Thus, there was no evidence of transmission from the group treated with viable micro-organisms to the Shelf control group.

Conclusion:

Under conditions of this study, *Beauveria bassiana* strain GHA was not toxic, pathogenic, or infective to male and female rats following administration of a single oral dose of about 1 x 10^8 CFU/animal (about 6 mg technical as/kg bw).

B.6.1.2.2.2 Acute inhalation toxicity, pathogenicity and infectiveness

Reference:	IIM 5.3.3/01
Report:	Barbera, P. W. (1993): Pulmonary toxicity/pathogenicity testing of Beauveria bassiana strain GHA following acute intratracheal challenge in rats. IIT Research Institute, Chicago, U.S.A. on behalf of Mycotech Corp. Unpublished Report No. L08433, Study no. 4. Pathology report provided by Pathology Associates, Inc., Chicago, U.S.A. on behalf of IIT Research Institute. TOX2006-872
Guidelines:	Currently, there is no OECD or EU guideline for studies of this type with micro-organisms. It is stated in the report that U.S. EPA 152A-13 was followed which is comparable to OPPTS Guideline 885.3150. Deviations: None
GLP:	Yes (self-certified in accordance with US 40 CFR 160)
General assessment:	The study is considered acceptable although a more comprehensive histological evaluation would have been very helpful.

Material and methods:

Young male and female CD rats (supplier: Charles River Labs., Portage/Michigan, U.S.A.) were used in this study receiving a single intratracheal application of *Beauveria bassiana* strain GHA (Lot No. 930210GHA-A). The target concentration was about 1 x 10^8 CFU per animal, based on the outcome of a pre-test in three male and three female rats employing the same concentration that did not cause mortality or overt clinical signs of toxicity over a two-day observation period.

A large number of rats was included since, in the main study, all groups comprised of 10 animals per sex and timepoint of sacrifice. A first treatment group (designated as "TS") received viable conidia at actual amounts of 1.01 x 10^8 CFU (males) or 1.05 x 10^8 CFU (females). A second group (KTS) was given killed *Beauveria bassiana* strain GHA spores at rates of 1.03 x 10^8 CFU (males) or 1.09 x 10^8 CFU (females) (autoclaved for 20 minutes at 121 ºC). Taking into account the mean body weight of male and female rats on the day of dosing and an amount of 8 x 10^{10} CFU per gram test substance, this would correspond to a dose of 5.7 mg technical a.i./kg bw in males and of 6.7 mg technical as/kg bw in females.

The rats which were allocated to these two groups as well as to the naïve control group (NC) were sacrificed by pentobarbital overdose on study days 0 (post dosing), 3, or 7. Although not clearly stated in the report, it is assumed that the control animals were administered the vehicle without spores by the intratracheal route. A so-called shelf control (SC) of 10 rats per sex was housed together with the TS group animals in the same room to allow for detection of a possible horizontal transfer of this micro-organism or parallel infection with other germs. These rats were apparently not treated and were sacrificed on day 14 by CO_2 asphyxiation, together with so-called "extra rats" (10 per sex from the NC, TS and KTS groups) that had been dosed and kept for possible replacement purposes and for future investigations if needed. However, it is obvious from the report that these extra rats from the groups other than SC were not examined for any parameter apart from body weight determination.

Food and fresh water were administered *ad libitum* and animals were housed up to two per cage at a temperature varying between 23 – 29 ºC and a relative humidity between 23 and 67 %. A 12 hour light/dark cycle was maintained throughout the study.

Following adaptation to the laboratory conditions, the rats were fasted for 17 - 25 hours and then given the test substance in a dosing volume of 0.1 ml directly into the trachea. Suspensions were prepared by adding of 1 g of the micro-organism (approx. 8 x 10^{10} CFU) to 80 mL of purified water containing 0.1 % Tween 80 and vortexing for one minute. Afterwards, the KTS groups dosing preparations were autoclaved. Before dosing, suspensions were vortexed again, allowed to settle for one minute and further diluted with purified water.

The rats were observed daily for clinical signs, behavioural changes and mortality. Body weights were monitored prior to randomisation, at the time of dosing (day 0) and on days 3, 7 and 14. At scheduled termination, gross necropsy was performed on all animals. Organ weights of brain, caecum, kidneys, liver, lungs and associated lymph nodes, and spleen were determined in seven rats per sex and timepoint of sacrifice. Heart, lungs and associated lymph nodes, thymus and trachea of the remaining three animals per group were subjected to histopathological examination.
In this study, again, microbial clearance was investigated. Processed samples obtained from blood and all the organs that had been removed from the body were plated on selective (DOA) and non-selective (SDAY) mycological media. Thus, 7 rats per group, sex and termination time were included. After at least 72-hour incubation at room temperature, microbial titer was determined by counting the colonies on duplicate spread plates.

Statistical evaluation of the microbial counts, body weights and organ weights was made by means of ANOVA and Dunnett´s test.

Findings:

There were no unscheduled deaths during the study and no clinical signs were noted that could be attributed to treatment.

Although this finding was not statistically significant, mean body weight gain in the treated groups was slightly depressed during the period following dosing. This reduction became apparent in males receiving viable conidia on day 3 and in females on day 7. However, during the second week, body weight gain values normalised suggesting an only weak and transient effect (see Table B.6.1.2-3). Interpretation of this finding is further complicated by the rather

large variance in individual values that was noted at the start of treatment already and continued throughout the study.
Surprisingly, in both sexes, shelf control animals showed a lower absolute body weight from the beginning of the experiment and over the whole study period. In females, however, mean body weight gain was extraordinarily high in this group during the first week of the post-observation period but virtually stopped during the second week. This pattern cannot be easily explained. In contrast to the other groups, the number of animals remained the same in this group over the study and thus, this finding cannot be attributed to certain animals being killed in the meantime. Nonetheless, in the lack of any detection of *Beauveria bassiana* in that untreated group and since there were no clinical signs of disease, the decline in body weight gain cannot be interpreted as an indication of transmission of the micro-organism to the shelf control.

Table B.6.1-3: Body weight gain (g) in CD rats following intratracheal application of *Beauveria bassiana* strain GHA; number of animals per group / mean values with (rounded) standard deviation

Group/ Interval	Males			Females		
	Days 0 – 3	**Days 0 - 7**	**Days 0 - 14**	**Days 0 – 3**	**Days 0 - 7**	**Days 0 - 14**
NC	30 / 34.94 ± 15.0	20 / 69.34 ± 16.3	10 / 133.12 ± 8.8	30 / 13.28 ± 12.8	20 / 25.42 ± 15.0	10 / 58.52 ± 13.1
SC	10 / 25.44 ± 33.8	10 / 69.51 ± 23.3	10 / 110.15* ± 15.7	10 / 30.67* ± 11.6	10 / 40.1* ± 7.8	10 / 41.1* ± 11.3
TS	30 / 19.64* ± 14.8	20 / 63.49 ± 20.5	10 / 122.54 ± 16.7	30 / 17.34 ± 13.6	20 / 13.28* ± 14.8	10 / 52.01 ± 7.5
KTS	30 / 24.23 ± 16.0	20 / 56.44 ± 20.8	10 / 123.29 ± 9.9	30 / 13.44 ± 12.6	20 / 24.23 ± 15.0	10 / 52.83 ± 12.0

NC naïve control; SC shelf control; TS treated with viable spores; KTS treated with killed spores
* $p < 0.05$ as compared to NC group, Dunnett´s test

Necropsy did not reveal any gross pathological lesions. However, absolute and relative weights of lungs and associated lymph nodes were markedly higher in the groups receiving viable and killed spores Mean values are given in Table B.6.1.2-4. Both sexes were affected. Thus, a treatment-related effect is likely although the differences were not always statistically significant. (It must be emphasised that statistical analysis was apparently confined to relative organ weights. Absolute organ weights were not considered.) The increase in lung weight was in line with the histopathological findings reported below. There were no changes in mean absolute or relative weight of other organs that could be attributed to treatment.

Table B.6.1-4: Absolute and relative weight of lungs and associated lymph nodes following intratracheal challenge with *Beauveria bassiana* strain GHA

Day post dosing	Parameter	Males			Females		
		NC	TS	KTS	NC	TS	KTS
0	Absolute weight (g)	1.11	1.08	1.13	0.98	0.99	1.05
	Relative weight (%)	0.53	0.49	0.53	0.50	0.54*	0.56*
3	Absolute weight (g)	1.15	1.62	2.00	1.07	1.70	2.07
	Relative weight (%)	0.51	0.70	0.88*	0.57	1.16*	0.91*
7	Absolute weight (g)	1.34	1.86	1.65	1.07	1.52	1.52
	Relative weight (g)	0.50	0.69*	0.61*	0.52	0.79*	0.74*

NC naïve control; TS treated with viable spores; KTS treated with killed spores
* $p < 0.05$ as compared to NC group, Dunnett´s test

Histopathology was confined to three animals per sex, dose group and sacrifice time. Obviously, only lungs were examined histologically although, according to the study report, more tissues had been preserved for histopathology. The most abundant findings in TS and KTS rats immediately after dosing were perivascular infiltration mainly by neutrophils and acute peribronchiolar inflammation. 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 edema were observed. Incidence and severity of the lesions appeared higher in the KTS than in the TS 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.

The sensitivity of the microbiological method of detecting viable spores was sufficiently high. 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 micro-organism to other parts of the body or to the shelf control group that was housed together with the TS animals in the same room. No test substance could be recovered from the KTS or NC groups.

Conclusion:

Following intratracheal administration to rats, *Beauveria bassiana* strain GHA was not infective. The micro-organism also proved not pathogenic and not toxic in terms of inducing an ongoing systemic disease process. The acute intratracheal LD_{50} was >1.01 x 10^8 CFU for males and >1.05 x 10^8 CFU for female rats (about 6 mg technical as/kg bw). Microbial clearance was complete by day 7 post dosing. However, there was evidence of a local reaction to treatment in the lungs as demonstrated by higher organ weights and histopathological findings, mainly inflammation. These changes may have also contributed to a transient reduction in body weight gain. Both sexes were affected and the lesions were still present on day 7. The study authors interpreted these changes as a normal mechanic response to the

injection of foreign material but the reaction was clearly adverse. Long-lasting local changes in the respiratory tract may alter the general state of health. It is not clear from that study how long the effects would persist and what would happen upon repeated challenge. Furthermore, the immune system was involved as demonstrated by cellular infiltration and, thus, potential sensitisation by inhalation cannot be excluded.

B.6.1.2.2.3 Intraperitoneal/subcutaneous single dose

Reference:	IIM 5.3.4/01
Report:	Barbera, P. W. (1993): Toxicity/pathogenicity testing of *Beauveria bassiana* strain GHA following acute intraperitoneal challenge in rats. IIT Research Institute, Chicago, U.S.A. on behalf of Mycotech Corp. Unpublished Report No. L08433, Study no. 5. TOX2006-873
Guidelines:	Currently, there is no OECD or EU guideline for studies of this type with micro-organisms. It is stated in the report that U.S. EPA guideline 152A-13 was followed which is comparable to OPPTS Guideline 885.3200 (not checked by the RMS). Deviations: Test animals were sacrificed by CO_2 instead of pentobarbital since this was considered a more appropriate means of sacrifice. This minor deviation does not compromise the scientific validity of this study
GLP:	Yes (self-certified in accordance with US 40 CFR 160)
General assessment:	The study is considered acceptable.

Material and methods:

A sample of *Beauveria bassiana* strain GHA was tested for toxicity in groups of 12 male and 12 female CD rats. The animals were treated with a target dose of 1×10^7 CFU that was given in 1 mL by intraperitoneal administration (TS group). Taking into account the mean body weight of male and female rats on the day of dosing and an amount of 8×10^{10} CFU per gram test substance, this would correspond to a dose of 0.54 mg technical as/kg bw in males and of 0.72 mg technical as/kg bw in females. An additional group of the same size (KTS) was treated with killed *Beauveria bassiana* strain GHA conidia at the same rate, and two untreated control groups, Naïve Control (NC, 12 rats per sex) and Shelf Control (SC, 6 per sex, housed in the same room as the treated animals and killed on study day 7), were also set up. Food and drink were administered *ad libitum* and animals were housed individually in wire cage between 23 – 28 ºC and at relative humidity between 30 and 70 %. A 12 hour light/dark cycle was maintained throughout the study.
Doses were prepared by weighing 1 g *Beauveria bassiana* strain GHA (Lot 930210GHA-A) into 80 mL purified water containing 0.1 % Tween 80, and mixed by vortexing for 1 minute. In the case of the KTS group, these suspensions were autoclaved for 20 minutes at 121 ºC to kill the organisms prior to dosing. Sterility of the KTS suspension was confirmed by plating out.

After treatment, three animals from the TS, KTS, and NC groups were sacrificed by carbon dioxide asphyxiation on days 0 and 3 whereas the remaining animals including all SC rats were killed on day 7. Among those sacrificed at study termination, only 3 rats per sex were used for further investigations since the other had been kept as "replacement animals" only.
The rats were checked daily for mortality and clinical signs. Body weights were determined prior to randomisation, at the time of dosing (day 0), and on days 3 and 7. At termination, all rats were subject to gross necropsy and organ weights were recorded at least for brain, caecum, kidneys, liver, lungs and associated lymph nodes, mesenteric lymph nodes and spleen.

Investigations for microbial clearance were performed by enumerations of fungi in blood, brain, caecum, kidneys, liver, lungs and associated lymph nodes, mesenteric lymph nodes and spleen and in lavage fluid from the peritoneal cavity. Processed samples were plated either on Sabouraud dextrose agar or on dodine oatmeal agar.

Findings:

There were no mortalities in the study. Neither clinical signs of toxicity, nor adverse gross pathological changes were observed in any group. Body weights or organ weights were not affected.

Beauveria bassiana strain GHA conidia were isolated only on day 0, and were found in the spleen, kidneys, liver and peritoneal cavity of male and female rats treated with living conidia. They were not found in any other group. By day 3, clearance of *Beauveria bassiana* strain GHA conidia from the body was established.

Conclusion:

In conclusion, *Beauveria bassiana* strain GHA was not toxic, pathogenic or infective when administered once by the intraperitoneal route to male and female CD rats. Microbial clearance was complete within three days after dosing. For micro-organisms, this route is of particular relevance since it might mimic conditions in which the natural protective barriers of the mammalian organism have been penetrated. Thus, it may be considered a worst-case situation with regard to infectivity and pathogenicity.

B.6.1.2.2.4 Published information on acute effects of *Beauveria bassiana* in mammals

To a certain extent, the results of the studies with strain GHA are supported by some further data on acute health effects of this fungal species that could be found by the RMS in the open literature even though, in general, information was scarce.

Melnikova and Murza (1980, TOX2007-478) reported the LD_{50} to be in excess of 1.1×10^{10} CFU/animal following single oral (intragastric) application and 2.2×10^{10} CFU/animal after intraperitoneal injection to rats. Rabbits even tolerated a very high amount of *Beauveria bassiana* when administered intravenously with an LD_{50} of more than 4×10^{10} CFU/animal. For the inhalation experiment, precise figures in terms of LC_{50} were not given but no distribution of the fungus from the lungs into other organs was observed and the lungs got completely rid of it within 4 - 5 days. Unfortunately, no experimental details were given in the publication and it is not known which strains of *Beauveria bassiana* were used at that time in

the former Soviet Union. This paper seems to provide the only available (although very brief) information regarding sensitisation by inhalation (and not intratracheal administration) of *Beauveria bassiana* spores. It was reported that some of the sensitised guinea pigs exhibited signs of anaphylaxis in an inhalation experiment. Furthermore, "hypersensitivity of blood neutrophils" was mentioned in this species but it is not clear what this finding exactly means. Perhaps, infiltration of neutrophils in lung tissue was observed, *i.e.*, a typical event in the first phase of an immunological reaction.

Semalulu *et al.* (1992, TOX2007-480) injected viable *Beauveria bassiana* spores of the "laboratory strain" GK2016 (not further described) in a 20 µL suspension into the quadriceps muscles of 20 adult CD-1 mice per sex. Each animal received an injection of a low dose (2 x 10^5 spores) into the right and was, in parallel, administered the high dose (2 x 10^8 spores) into the left quadriceps muscle. Groups of five mice (2 males and 3 females or *vice versa*) were killed at 8 different times ranging from 12 hours to 28 days post dosing and examined for histological lesions around the injection sites and for the presence of the spores and possible multiplication of the micro-organism. Lymph nodes next to the injection sites were also dissected and subjected to histopathology.
Intramuscular injection caused a strong local reaction that was more pronounced around the high dose injection sites than at the sites of low dose administration. At 12 hours post dosing, severe edema and focal hyaline necrosis of muscle fibres were observed. Infiltration of neutrophils in the interfascicular connective tissue suggested an immune response. Within the next two days, a mild to moderate focal suppurative myositis developed and degeneration of muscle fibres was noted. From day 3 onwards, there was infiltration of macrophages more and more replacing that by neutrophils. By day 7, the reaction had converted to focal granulomatous changes and first signs of regeneration of muscle fibres were seen that apparently progressed over the subsequent weeks. At the low dose injection sites, no inflammation was apparent on day 14 any more but around the high dose injection sites, some lesions were still visible after 28 days.
Following high dose application, spores were present around the injection sites over the whole study period but viable fungus was not detected from day 3 onwards. At the low dose sites, viable spores were seen only at 12 hours post dosing. Later on, the spores became more and more degenerated. Spores were found in the popliteal lymph nodes after 7, 14 or 21 days but had been taken up by macrophages already and there was no evidence of replication of the fungus.
Reviewing some publications on this micro-organism in the discussion section of this paper, the authors emphasised that *Beauveria bassiana* was not infectious or pathogenic but might act as an allergen.

B.6.1.2.3 Genotoxicity testing

An Ames test for point (gene) mutations has been performed with *Beauveria bassiana* strain GHA that is described in detail below. This study did not reveal evidence of genotoxicity. Further studies on mutagenicity were not submitted but this is not considered a critical data gap because of the following considerations:
A genotoxic potential of fungi, if occurring, is assumed to be due to mycotoxins or other metabolites. It is known that some strains of *Beauveria bassiana* can produce two cyclic depsipeptides, *i.e.*, beauvericin and bassianolide. The few available published studies with these biochemicals suggest that they may contribute to the insecticidal activity of this micro-organism. According to the notifier, Laverlam International Inc. (*i.e.*, the manufacturer) had

analysed over 40 production lots of *Beauveria bassiana* strain GHA and did not find either biochemical in any of the batches (Bradley, 1993, TOX2006-898). However, when analysing two laboratory batches of that strain, levels of about 50 ppm of both substances were found in one of them (see B.6.1.2.2 and Volume 4).
Mutagenic activity of these metabolites is unlikely because of the large molecular weight of beauvericin and bassianolide. The larger a molecule, the more difficult is it to penetrate the mammalian cell membrane, transverse the cytoplasm, and cross the nuclear membrane. The molecular weights of beauvericin and bassianolide are about 781 and 878 Dalton, respectively. Exogenous molecules of this size and complex stereoconfiguration should have great difficulty crossing the cell membrane. They should be unable to reach the DNA within the nucleus, a step essential for the expression of genotoxicity. No cyclic depsipeptide has been identified as a mutagen or as a genotoxic carcinogen. Essentially all biochemical mutagens have molecular weights below 500 Dalton.
Moreover, at least beauvericin proved negative in an Ames test in five *Salmonella typhimurim* tester strains (TA97, 98, 100, 102, and 1535) with and without metabolic activation at concentrations of up to 500 µg/plate (Fotso and Smith, 2003, 1689977).

B.6.1.2.3.1 *In vitro* studies

Reference: IIM 5.3.4/5.3.6

Report: Sokolowski, A. (2006): Salmonella typhimurium and Escherichia coli reverse mutation assay with Beauveria bassiana Strain GHA conida spores. Cytotest Cell Research GmbH (RCC-CCR), Rossdorf/Germany on behalf of Laverlam Int. Corp., Study no. 923900.
1679372

Guidelines: "Ninth Addendum to OECD Guidelines for Testing of Chemicals", Section 4, No. 471: "Bacterial Reverse Mutation Test", adopted July 21, 1997

"Commission Directive 2000/32/EC, L1362000, Annex 4D", dated May 19, 2000

Deviations: none

GLP: Yes

General assessment: The study is considered acceptable.

Material and methods:

Beauveria bassiana strain GHA conida spores were assessed for their potential to induce gene mutations in the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, and TA 100, and the *Escherichia coli* strainWP2 uvrA.

The assay was performed in two independent experiments both with and without liver microsomal activation. Each concentration and the controls were tested in triplicate. The test item was tested at the following concentrations:

Pre-experiment/experiment I: 3, 10, 33; 100; 333; 1000; 2500; 5000 µg/plate
Experiment II: 1; 3, 10, 33; 100; 333; 1000; and 2500 µg/plate
The plates incubated with the test item showed normal background growth up to the highest investigated concentration with and without metabolic activation in both independent experiments.

Findings:

No toxic effects, evident as a reduction in the number of revertants, occurred in the test groups with and without metabolic activation.
No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with *Beauveria bassiana* strain GHA conidia spores at any dose level, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance.
Appropriate reference mutagens were used as positive controls and showed a distinct increase of induced revertant colonies**.**

Conclusion:

Beauveria bassiana strain GHA 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 in this *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay.

B.6.1.2.4 Cell culture study

Not applicable. A study of that type is only required for viruses or for other infectious agents with intracellular replication but not for micro-organisms such as *Beauveria bassiana*.

B.6.1.2.5 Information on short-term toxicity and pathogenicity

No study with repeated administration of *Beauveria bassiana* strain GHA was submitted. A search for information on short-term effects of *Beauveria bassiana* (irrespective of the strain) in the open literature was not successful. However, in a Chinese long-term study on rats and mice (Song, 1989, TOX2007-481, see B.6.2.1), inhalative exposure resulted in effects that were similar to those observed in the acute intratracheal study and that confirmed the allergenic potential to the respiratory tract. Thus, there is no need to require a subacute or subchronic inhalation study. The other routes of exposure are of no oncern because no adverse effects in terms of infectivity, pathogenicity or toxicity were observed in the acute studies. Furthermore, clearance of the micro-organism from the mammalian host was rapid.

B.6.1.2.6 Proposed treatment: First aid measures, medical treatment

There are no specific first aid measures applicable. According to the available data concerning infectivity, pathogenicity and toxicity and taking into account the rather low exposure, 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 because of aspiration.
In humans, eye, lung and disseminated infection with *Beauveria bassiana* have been occasionally reported (see section B.6.1.1.1.3). In the unlikely case of such events following contact with strain GHA, specialist´s expertise and treatment with anti-mycotic drugs will be needed.

B.6.1.3 Summary and conclusions of Tier I studies

Based on all available original studies with strain GHA and publications on acute effects of *Beauveria bassiana* (irrespective of the strain used), it can be concluded that strain GHA, as the whole species, does not cause infections in mammals and is not pathogenic in terms of inducing a systemic disease process. This has been proven in experiments employing different routes of exposure such as the oral, inhalative (intratracheal), and even intraperitoneal or intramuscular. In contrast, local pathological changes and transient effects on body weight have been observed following intratracheal challenge of rats with the strain GHA (Barbera, 1993, TOX2006-872) as well as subsequent to intramuscular injection to rats (Semalulu *et al.*, 1992, TOX2007-480). Thus, some toxicity may occur and seems to be route-dependent. The lung seems to be particularly sensitive. This assumption is further substantiated by data from the open literature (Fromtling *et al.*, 1979, TOX2007-472; Melnikova and Murza, 1980, TOX2007-478; Song, 1989, TOX2007-481). At least partly, the findings in the intratracheal study on rats may be interpreted as an immunological response to the micro-organism. This is well in line with reports on allergic reactions following acute inhalative exposure of guinea pigs (Melnikova and Murza, 1980, TOX2007-478) or chronic inhalative challenge of rats and mice (Song, 1989, TOX2007-481, see section B.6.2.1). Based on these findings, and taking into account the evidence of allergenicity in humans (e.g., Beaumont *et al.*, 1985, TOX2007-480; Westwood *et al*., 2005, TOX2007-482, see section B.6.1.1), the negative outcome of the Buehler test with strain GHA (Findlay, 1998, TOX2006-870) is not sufficient to exclude sensitising properties. Furthermore, inhalation seems to be more critical than sensitisation by dermal contact and would not be elucidated by a Buehler test. Therefore, classification and labelling of this micro-organism as a potential inhalatory and skin allergen (Xn, R42/43) is considered appropriate.

A genotoxic potential of strain GHA is not expected because metabolites of concern do not occur at high concentrations. *Beauveria bassiana* strain GHA as well as its metabolite beauvericin proved both negative in the Ames test.

Information on short-term toxicity (*i.e.,* a study with repeated administration) for strain GHA and for *Beauveria bassiana* in general is lacking and this might be considered a critical data gap. According to Annex IIM of Directive 91/414/EC, a study of this type belongs to the basic requirements for micro-organisms but may be waived under certain circumstances. In principle, *Beauveria bassiana* strain GHA should be a candidate micro-organism for waiving because no evidence of infectivity was obtained in the acute studies. Furthermore, *Beauveria bassiana* in general is expected to die at temperatures above 35 ºC, and, thus, cannot survive in mammalian tissues although experience in humans seem to partly contradict this assumption because cases of disseminated infections were reported (for details, see B.6.1.1.4). Because treatment-related systemic and local effects have occurred in the acute study with intratracheal administration to rats and were not fully reversible until termination, this route of exposoure is considered the most critical. However, in a long-term study on rats and mice,

inhalative exposure resulted in effects that were similar to those observed in the acute intratracheal study and that confirmed the allergenic potential to the respiratory tract. Thus, there is no need to require a subacute or subchronic inhalation study since no new information is to be expected.

B.6.2 Tier II - The active micro-organism

B.6.2.1 Specific toxicity, pathogenicity and infectiveness (OECD IIM 5.5.1)

The only toxicological study that was performed and submitted by the notifier in addition to the package of basic studies was an acute dermal study with strain GHA in rabbits (Johnson, 1993, TOX2006-874). In the open literature, a further specific study was found (Ishibashi *et al.*, 1987, TOX2007-474) dealing with possible damage to the rabbit eye. This examination was conducted because of anecdotal reports on keratitis and other eye infections in humans (see section B.6.1.1.3). Furthermore, very brief information on the effects of long-term inhalative exposure of rats and mice to *Beauveria bassiana* is available (Song, 1989, TOX2007-481). The hypothesis of a particular sensitivity of the lungs is also supported by observation in reptiles (Fromtling *et al.*, 1979, TOX2007-472). In the cited publications, however, the strain was not characterised.

Dermal study in rabbits

Reference:	IIM 5.5/01
Report:	Johnson, W.D. (1993): Acute dermal toxicity study of *Beauveria bassiana* GHA in rabbits, IIT Research Institute, Chicago, U.S.A. on behalf of Mycotech Corp. Unpublished Report No. L08433, Study no. 3. TOX2006-874
Guidelines:	40 CFR Section 158, Guideline 152A-11, which is claimed to be comparable to US EPA OPPTS 885.3100 (not checked by the RMS). Deviations: None.
GLP:	Yes (self-certified in accordance with US 40 CFR 160).
General assessment:	The study is considered acceptable.

Material and methods:

Two grams of the undiluted test material, a white powder containing 8.0 x 10^{10} CFU of *Beauveria bassiana* strain GHA (Lot number 930210GHA-A)/g was administered for 24 hours to the shaven backs of five male and five female New Zealand White rabbits. Thus, the whole amount of viable microorganism applied was 1.6 x 10^{11} CFU. Taking into account the mean body weight of male and female rabbits on the day of dosing, this amount corresponded to a mean dose of approx. 470 mg technical a.i./kg bw in males and of approx. 630 mg

technical as/kg bw in females with an individual range between 440 - 740 mg technical material/kg bw, depending on the body weight of the individual animals.

Animals were individually housed in stainless–steel cages between 21 – 23 °C and at relative humidity between 36 – 53 %. A 12 hour light/dark cycle was maintained throughout the study.

Twenty four hours prior to testing, the fur had been clipped from each animal`s trunk (approximately 10 % or 240 cm^2 of dorsal body surface area). The test sites were covered with surgical dressing and an elastic adhesive bandage This was left in place for 24 hours. The dressings were then removed, and the treated site swabbed with dilute saline. Rabbits were observed at frequent intervals immediately following dosing and then once per day for 14 days.

Any skin reactions were graded according to the Draize method. Body weights were recorded regularly. All animals were sacrificed on day 14 .

Findings:

There were no deaths during the study period and no signs of systemic toxicity were recorded in any animal during the study. However, it must be emphasised that necropsy was not performed. An increase in body weight was not to be expected because the study was run in adult animals. Actually, a very small increment was observed in female rabbits whereas in males at least no substantial body weight losses were noted.
Local effects indicating slight dermal irritation comprised erythema appearing in all animals within 30 - 60 minutes after removal of the wrappings. Erythema had dissolved in male rabbits by day 11 and in females by day 12 but red spots at the application site still persisted in five rabbits (3 males and 2 females) at study termination.

Conclusion:

The acute dermal LD_{50} in this study in rabbits was > 1.6 x 10^{11} CFU/animal (corresponding to about 470 or 630 mg as/kg bw). No evidence of infectivity, pathogenicity or systemic toxicity was obtained, however, slight dermal irritation was observed.

Pathogenicity to rabbit eye (published data)
Ishibashi *et al.* (1987, TOX2007-474) used rabbits to evaluate the pathogenicity of *Beauveria bassiana* and of *Candida albicans* in cornea. Both fungi were isolated from patients (diagnosed with keratitis or acute cutaneous candidiasis, respectively), inoculated on Sabouraud glucose agar and cultivated at 25 °C. Yeast cells were suspended in physiological saline, counted and adjusted to concentrations of 1.0 x 10^6 cells/mL (both) and 5.0 x 10^6 cells/mL (only *B. bassiana*). 26 male rabbits were divided at random into two groups. Both eyes of all rabbits received a subconjunctival injection of dexamethasone sodium phosphate every day for five days. The last day rabbits were anaesthetised with intravenous sodium pentobarbital and oxybuprocain was instilled topically. Using 27-gauge needles, a pocket was created approximately in one-half thickness deepness of the cornea in both eyes of all animals. Right eyes of all animals received 10 µL C. albicans suspension (i.e. 1.0 x 10^4 cells) into the pockets. One group received 10 µL of lower concentrated suspension (i.e. 1.0 x 10^4 cells) of *B. bassiana* into the left cornea, the other group received 10 µL of higher concentrated suspension (i.e. 5.0 x 10^4 cells). Eyes were examined with split lamp every day for three weeks. The severity of the inflammatory reaction of the cornea, anterior chamber, and iris was

evaluated. After clinical corneal infection developed, direct examination and cultures of specimens from lesions were performed to confirm the presence of the micro-organisms. On days 5, 10 and 15 after inoculation, one rabbit from each group was selected at random and sacrificed for histopathological examination. Eyes were enucleated, fixed in formaldehyde and mounted in paraffin. Sections were stained with periodic acid-Schiff and haematoxylin eosin for examination.

All 26 eyes treated with *C. albicans* developed clinical corneal infections. The microscopic examination of corneal biopsies from these eyes showed abundant fungal elements in the stroma. *C. albicans* was isolated in cultures of all right eyes. Seven of the 13 left eyes treated with the lower concentrated suspension of *B. bassiana* developed clinical corneal lesions. Direct examination of biopsy samples showed fungal elements in corneal stroma and the micro-organism was isolated in cultures. Eleven of 13 left eyes treated with the higher concentrated suspension developed clinical infections. Direct examination of corneal stroma showed the presence of fungal hyphae and *B. bassiana* was isolated in cultures. Severity of keratitis peaked about 12 to 14 days after treatment with *C. albicans*. Clinical features on day 14 included large white corneal ulcers, slight corneal haze, large hypopyon, neovascularisation from the corneoscleral limbus, and injection in the iris. Eyes treated with lower dose of *B. bassiana* showed minimal inflammatory reaction between days 10 and 18 with a peak on days 12 to 14. The clinical features included small corneal lesions and slight injection of the iris. Eyes treated with the higher dose of *B. bassiana* showed minimal inflammatory reaction from day 7 which increased in severity up to a peak on days 12 to 14, after which it gradually subsided. Small corneal ulcers, slight corneal haze, and slight injection of the iris were found on day 14. Histological examination on day 5 showed growth of *C. albicans* in the cornea and its surrounding by minimal infiltration of inflammatory cells, although the fungus did not invade the anterior chamber. *B. bassiana*, in contrast, was found only at the injection site and showed no growth at this time of examination. On the tenth day, there was considerable fungal growth in the cornea and invasion of the anterior chamber in eyes with *C. albicans* keratitis. Inflammatory cells had infiltrated moderately into the cornea, and hypopyon was seen in the anterior chamber. In eyes with *B. bassiana* keratitis, the fungus was present only in the middle cornea and showed sparse growth. In eyes, 15 days after treatment with *C. albicans*, a large number of inflammatory cells had infiltrated the cornea, fungal elements were found in the infiltrates as debris. In *B. bassiana* keratitis, the fungus showed sparse to moderate growth only in the middle cornea, and there was very little inflammatory reaction around the fungal elements. Eyes from both groups treated with *B. bassiana* gave similar results during histological examination and were therefore not reported separately.

Long-term inhalation study in rodents (published data)

Song (1989, TOX2007-481) reported a long-term inhalation study with *Beauveria bassiana* in rats and mice. As the article was written in Chinese, only the abstract was available and is given here: “113 lung specimens from rats and mice were observed under both LM and TEM, after inhalation of gonidiospores of *Beauveria bassiana* for 18 months 78.8 % of the 113 cases developed chronic interstitial pneumonia (IP). There were desquamative pneumonitis mainly with macrophage; granuloma with multinucleate giant cells or fibrosis, and localised pulmonary edema. These lesions were firstly described to be caused by the spores here. It was considered that IF lesions might be related to types III, IV hypersensitivity reaction. The authors emphasised that these lesions might be similar to those observed in farmer's lung or extrinsic allergic alveolitis (EAA).”

Pulmonary infections in reptiles (published data)
Fromtling et al. (1979, TOX2007-472) reported an outbreak of pulmonary mycosis caused by *Beauveria bassiana* among captive American alligators (*Alligator mississipiensis*) in a zoo. Following a drop in temperature because of a heating system failure in a hibernation grotto (decrease by 8 ^{o}C for 12 hours), three of four animals died within the next 9 months. Pathological and microbiological examinations revealed a severe systemic mycosis that affected the lungs and the pleura but was strictly confined to the thoracic cavity. The authors also referred to a paper reporting pulmonary mycosis due to infection with *Beauveria bassiana* in giant tortoises (Georg et al., 1962, not available to the RMS).

B.6.2.2 In vivo studies in somatic cells (OECD IIM 5.5.2)

No information provided. Since there is no genotoxic concern on strain GHA of *Beauveria bassiana* (for justification, see section B.6.1.2), no studies should be required.

B.6.2.3 Genotoxicity - In vivo studies in germ cells (OECD IIM 5.5.3)

No information provided. Since there is no genotoxic concern on strain GHA of *Beauveria bassiana* (for justification, see sections B.6.1.2), no studies are required.

B.6.2.4 Evaluation of Metabolites

In open literature, *Beauveria bassiana* is described to produce a range of different metabolites: beauvericin, bassianolide, bassiacridin, beauveriolides, beauverolides, bassianin, tenellin, oxalic acid and oosporein. Production of these metabolites depends on the culture conditions and possibly on the strain. Besides to beauvericin (< LOQ of 50 ppm), oosporein (not detected, no LOQ given), bassianolide (< LOQ of 50 ppm) and bassianin/tenellin (cultures of GHA show not the yellow colour of these metabolites) there is no information available if these metabolites can be formed in strain GHA (Volume 4).
Production of some metabolites is associated with infestation of a host. It seems that high amounts of metabolites can be produced after contact with the target pest (Section B.9.1).

There is only little information available on the toxicological properties of metabolites and how they might contribute to the insecticidal activity.
Zimmermann (2007, 1689994) named beauvericin “the most important compound which was reported first for *B. bassiana*”. As a precaution and due to the lack of reliable data, it is assumed that the metabolites act additively.

The acute toxicity studies with *Beauveria bassiana* strain GHA with oral, dermal or i.p. administration were conducted with a technical material that contained approx. 50 ppm beauvericin. In these studies no adverse effects were noted. The observed effects following intratracheal application are unlikely to be due to beauvericin.

Mutagenicity of beauvericin (Fotso & Smith, 2003, 1689977) was tested in an Ames test with *Salmonella typhimurium* strains TA97, TA98, TA100, TA102, TA1535 (provided by Molecular toxicology, Boone, NC, USA). Plate incorporation and preincubation technique were used. S9-mix from Aroclor 1254-induced male rats was used. Sodium azide, fenaminosulf, 2-aminofluorene served as positive control compounds.

In plate incorporation test with doses of up to 500 µg/plate, beauvericin was not mutagenic with and without S9-mix. Authors stated, that the positive control compounds increased revertant frequency (data were not shown). Preincubation test showed no increase of revertant number in a concentration of 20 µg/plate (data were not shown). Toxicity was assessed in *Vibrio fisheri* and gave an EC_{50} for bioluminescence of approx. 70 µg/mL.

Furthermore, beauvericin induced apoptosis or cell death in a range of various cultured cell. *In vitro* studies indicated a potential to alter cationic levels. In mouse macrophages and rat liver microsomes, acylcholesterol ester formation by acyl-CoA-cholesterol acyl transferase was inhibited.

The available studies cannot be used to derive safe levels for metabolite exposure. Therefore, it is proposed to use the concept of the threshold of toxicological concern (TTC) (Munro et al., 1999;1689983, Kroes et al., 2000 (1689981) and 2004 (1689982). The compounds are neither non-essential metals, metal containing compounds, or polyhalogenated-dibenzodioxins, -dibenzofurans or -biphenyls nor is there a concern for potential genotoxicity. The TTC of 1.5 µg/d (i.e., 0.02 µg/kg bw/d, calculated with a bodyweight of 75 kg) is considered a safe exposure for the sum of all metabolites (due to the presumed additive action). If the daily exposure with the metabolites does not exceed this level, metabolites are not expected to be a safety concern. In this case, it is not necessary to require further toxicological studies which would allow to derive NOAELs.

There is only an analytical method for beauvericin available. Hence, it is proposed to use the beauvericin content as an indicator for the presence of the other metabolites.

Evaluation of operator and consumer exposure was performed with content of (the sum of) the metabolites in the technical material of 50 ppm. For the specification, maximum levels for individual metabolites have to be given. Therefore, 50 ppm was divided by the number of metabolites (9) and the result was rounded to 5 ppm. Due to the lack of individual analytical methods for all metabolites, it is proposed to specify only beauvericin (maximum level in the technical material: 5 ppm).

B.6.2.5 Summary and conclusions of Tier II studies

The acute dermal study with strain GHA revealed evidence of slight irritation but proved the absence of infectivity, pathogenicity and toxicity when applied by this route.

A certain potential to damage the cornea was confirmed in rabbits after observations in humans had already suggested such properties. However, no special risk for operators is anticipated since adverse effects on human eyes occurred only after mechanical injury of the cornea allowing the fungus to invade. Nonetheless, the cornea seems to be a tissue that provides suitable conditions for survival and at least limited growth of this micro-organism.

Results of a long-term inhalation study in rats and mice might support the assumption that *Beauveria bassiana* might be an inhalation allergen.

Fatal chronic pulmonary mycoses due to infection with *Beauveria bassiana* occurred in crocodiles and tortoises. Of course, evidence of infection with that micro-organism in poikilothermic species does not contribute to human health risk assessment but, again, points

to the lungs as a potential target of adverse effects of *Beauveria bassiana*. It seems that the fungus might find suitable conditions for colonisation and growth in lung tissue confirming similar observations in laboratory animals as well as in humans.

No information concerning mutagenicity *in vivo* has been submitted. But since there is no concern for strain GHA, this is not considered a data gap.

In open literature, *Beauveria bassiana* is described to produce a range of different metabolites: beauvericin, bassianolide, bassiacridin, beauveriolides, beauverolides, bassianin, tenellin, oxalic acid and oosporein. Production of some metabolites is associated with infestation of a host. It seems that high amounts of metabolites can be produced after contact with the target pest. Due to the lack of information on the toxicological properties of the metabolites, it is proposed to use the concept of the threshold of toxicological concern. Hence, a daily exposure to all metabolites (i.e., the sum of them) of 1.5 µg/d (i.e., 0.2 µg/kg bw/d) is considered a safe exposure. Occupational and consumer exposure assessment was conducted with a content of (the sum of) the metabolites of 50 ppm in the technical material. As there are 9 metabolites, 50 ppm is divided by the number of metabolites and the result is rounded to 5 ppm.
As there is only an analytical method for beauvericin available, its level is considered an indicator for the presence of the other metabolites. Hence, only beauvericin needs to be specified in the technical material (maximum level of 5 ppm).

B.6.3 Summary of mammalian toxicity, pathogenicity and effectiveness and overall evaluation of the active micro-organism (OECD IIM 5.6)

The entomopathogenic fungus *Beauveria bassiana* is a rare human pathogen since, in very few cases, it was isolated from eye infections, from pulmonary disease or, in immunocompromised patients, from disseminated infections. Animal data suggest that both corneal and lung tissues provide suitable conditions for survival and even replication of this micro-organism that is otherwise considered not to grow at temperatures above 35 ^{o}C. It is not known whether these pathogenic properties can be attributed to certain strains since such an information is usually lacking in the case reports. Because eye infections were observed after mechanical damage to the cornea only and systemic disease was apparently confined to severely ill people under immunosuppression, the clinical evidence that infections in humans may occur, is of limited relevance for human health risk assessment of strain GHA with regard to its intended use in plant protection. More important is the body of information suggesting allergenicity. Based on experience and tests in humans as well as on studies in laboratory animals, *Beauveria bassiana* in general should be considered a potential sensitiser by inhalation. Furthermore, skin sensitising properties of this micro-organism cannot be excluded.

A number of acute studies for infectivity, pathogenicity and toxicity of the strain GHA was submitted using the oral, intratracheal, intraperitoneal, and dermal routes. It should be noticed that these studies were performed with a batch that contained relatively large amounts of the metabolites beauvericin and bassianolide and, thus, to a certain extent, reflect worst-case conditions. In addition, a Buehler test for skin sensitisation was provided. The available original studies in laboratory animals are summarised Table B.6.3-1.

Table B.6.3-1: Summary of acute studies with *Beauveria bassiana* strain GHA

Annex II point	Study/Route/ Method	Species	Dose per animal	Results	Conclusion	Reference
IIM 5.3.1	Skin sensitisation (Buehler test 3 inductions)	Guinea pig	80 mg (approx. 8×10^9 CFU)	Negative (not sensitising)	No final conclusion can be drawn	Findlay (1998, TOX2006-870)
IIM 5.3.2	Oral gavage	Rat	approx. 1×10^8 CFU	No evidence of toxicity, pathogenicity or infectivity; rapid clearance (by day 3 post dosing)	$LD_{50} > 1 \times 10^8$ CFU; not to be classified, no concern	Barbera (1993, TOX2006-871)
IIM 5.3.3	Intratracheal	Rat	approx. 1×10^8 CFU	No evidence of infectivity but local effects on the lungs (inflammation/immune reaction, organ weight increase), transient reduction in body weight gain; clearance complete by day 7 post dosing	$LC_{50} > 1 \times 10^8$ CFU; evidence of some inhalation toxicity, sensitisation by inhalation cannot be excluded	Barbera (1993, TOX2006-872)
IIM 5.3.4	Intraperitoneal	Rat	approx. 1×10^7 CFU	No evidence of toxicity, pathogenicity or infectivity; rapid clearance (by day 3 post dosing)	$LD_{50} > 1 \times 10^7$ CFU; not to be classified, no concern	Barbera (1993, TOX2006-873)
IIM 5.5.1	Dermal	Rabbit	1.6×10^{11} CFU	No systemic effects but signs of slight but persisting local irritation	$LD_{50} > 1.6 \times 10^{11}$ CFU; not to be classified, no concern	Johnson (1993, TOX2006-874)

The results of these studies suggest that strain GHA was not infective by any route and not pathogenic in terms of inducing a systemic disease. Clearance from the body was rapid and virtually complete. In contrast, local pathological changes in the lungs, organ weight increase. and transient effects on body weight have been observed following intratracheal challenge. Thus, some toxicity may occur and seems to be route-dependent with the lung being the most sensitive target. This assumption is further substantiated by published data although apparently other strains were tested.

At least partly, these findings are in line with the assumption of an immunological response to the micro-organism. This is further supported by reports from the literature on allergic reactions following acute inhalative exposure of guinea pigs or chronic inhalative challenge of

rats and mice. The negative outcome of a Buehler test with only three inductions is not sufficient to exclude sensitising properties of strain GHA. Furthermore, inhalation seems to be more critical than sensitisation by dermal contact and would not be elucidated by a Buehler test. Thus, classification and labelling (Xn, R42/43) of strain GHA is needed.

Beauveria bassiana is known to produce a wide variety of toxic compounds. However, the three secondary metabolites of biggest concern, *i.e.*, bassianolide, beauvericin, and oosporein, have usually not been detected in liquid cultures of strain GHA or in the plant protection product. As reported elsewhere in the DAR, the amount of beauvericin and bassianolide was in most batches below 5 ppm. In the batch (lot) 930210 that was used in the single dose studies for infectivity, pathogenicity and toxicity, in contrast, higher concentrations of both metabolites (48 ppm of beauvericin and 51 ppm of bassianolide) were detected. Thus, possible toxic effects of these metabolites were at least partly covered by the available experimental data. Since a much lower limit for these substances is proposed, no risk to human health is anticipated.

A genotoxic potential of strain GHA is not expected because there is no production of metabolites of concern at higher concentrations. This assumption was supported by the negative outcome of Ames tests with both the micro-organism itself and the metabolite beauvericin.

A short-term study to investigate infectivity, pathogenicity and toxicity of strain GHA under conditions of repeated exposure is lacking. However, taking into consideration that the inhalative route was the by far most critical (based on the outcome of the acute studies), a published long-term inhalation study with *Beauveria bassiana* on rats and mice (strain not specified) was regarded as sufficient proof that the effects were similar to those observed after acute challenge. Pulmonary toxicity and sensitisation by inhalation were confirmed. No further information is expected from a short-term study with strain GHA and, thus, this requirement may be waived.

In open literature, *Beauveria bassiana* is described to produce a range of different metabolites: beauvericin, bassianolide, bassiacridin, beauveriolides, beauverolides, bassianin, tenellin, oxalic acid and oosporein. Production of some metabolites is associated with infestation of a host. It seems that high amounts of metabolites can be produced after contact with the target pest. Due to the lack of information on the toxicological properties of the metabolites, it is proposed to use the concept of the threshold of toxicological concern. Hence, a daily exposure to all metabolites (i.e., the sum of them) of 1.5 µg/d (i.e., 0.2 µg/kg bw/d) is considered a safe exposure. Occupational and consumer exposure assessment was conducted with a content of (the sum of) the metabolites of 50 ppm in the technical material. As there are 9 metabolites, 50 ppm is divided by the number of metabolites and the result is rounded to 5 ppm.
As there is only an analytical method for beauvericin available, its level is considered an indicator for the presence of the other metabolites. Hence, only beauvericin needs to be specified in the technical material (maximum level of 5 ppm).

B.6.4 Effects on human health - The preparation (OECD IIIM 7)

BotaniGard 22 WP is a wettable powder formulation (WP) containing *Beauveria bassiana* strain GHA (pure 220 g/kg or 4.4 x 10^{13} CFU/kg). The preparation is intended for use on ornamentals and vegetables in greenhouses.

A summary of the results of the acute toxicity studies including irritancy and skin sensitisation can be found in Table B.6.4-1.

Table B.6.4-1: Summary of toxicity study results obtained with BotaniGard 22 WP

Parameter	Test substance	Species	Results	Danger symbol, R-phrase	Reference
Acute oral LD_{50}	Mycotrol WP9616b (BotaniGard 22 WP)	Rat	> 5000 mg/kg bw	--	Johnson, W. D. (1997) TOX2006-875
Acute inhalative LD_{50}		Not submitted (*Beauveria bassiana* strain GHA: Intratracheal study, LC_{50} rat >1 x 10^8 CFU animal; evidence of some inhalation toxicity, sensitisation by inhalation cannot be excluded)		--	See B.6.3
Acute dermal LD_{50}		Not submitted (*Beauveria bassiana* strain GHA LD_{50} rabbits > 1.6 x 10^{11} CFU/animal, no systemic effects but signs of slight but persisting local irritation)		--	See B.6.3
Acute skin irritation	Mycotrol (BotaniGard 22 WP)	Rabbit	Non irritant	--	Findlay, J. (1999) TOX2006-876
Acute eye irritation	Mycotrol WP9616b (BotaniGard 22 WP)	Rabbit	Non irritant	--	Johnson, W. D. (1997) TOX2006-877
Skin sensitisation (Buehler test with 3 inductions)	BotaniGard 22 WP	Guinea pig	Sensitising	R 43	Arcelin, G. (2006) 1679375

BotaniGard 22 WP is of low acute toxicity following the oral route of exposure. The rat oral LD_{50} is higher than 5000 mg/kg bw. Based on the negligible acute dermal toxicity of the active substance and the co-formulants and since the micro-organism does not pass through the skin barrier an acute dermal study was not carried out for reasons of vertebrate protection. The conditions for reporting the inhalation toxicity of the plant protection product according to Directive 94/79/EC, amending Directive 91/414/EEC are not fulfilled and hence testing for inhalation toxicity is not relevant and has not been conducted. Even if the inhalation toxicity of the active substance in the rat is taken into consideration a low acute inhalation toxicity is predicted. In acute irritation tests with BotaniGard 22 WP the formulation was not irritant to the skin or to eyes in rabbits. Based on the findings in a non-adjuvant sensitisation test in guinea pigs and in accordance to Commission Directive 2001/59/EC, BotaniGard 22 WP has to be classified and labelled as a skin sensitiser.

B.6.4.1 Basic acute toxicity studies - The preparation (OECD IIIM 7.1)

BotaniGard 22 WP is a wettable powder formulation (WP) containing *Beauveria bassiana* strain GHA (pure 220 g/kg or 4.4 x 10^{13} CFU/kg). The preparation is intended for use on ornamentals and vegetables in greenhouses.

A summary of the results of the acute toxicity studies including irritancy and skin sensitisation can be found in Table B.6.4-2.

Table B.6.4-2: Summary of acute toxicity of BotaniGard 22 WP

Parameter	Test substance	Species	Results	Danger symbol, R-phrase	Reference
Acute oral LD_{50}	Mycotrol WP9616b (BotaniGard 22 WP)	Rat	> 5000 mg/kg bw	--	Johnson, W. D. (1997) TOX2006-875
Acute inhalative LD_{50}		Not submitted (*Beauveria bassiana* strain GHA: Intratracheal study, LC_{50} >1 x 10^8 CFU; evidence of some inhalation toxicity, sensitisation by inhalation cannot be excluded)		--	See B.6.1.2.2.2
Acute dermal LD_{50}		Not submitted (*Beauveria bassiana* strain GHA LD_{50} rabbits > 1.6x 10^{11} CFU/animal, no evidence of infectivity, pathogenicity or systemic toxicity, slight dermal irritation)		--	See B.6.2.1
Acute skin irritation	Mycotrol (BotaniGard 22 WP)	Rabbit	Non irritant	--	Findlay, J. (1999) TOX2006-876
Acute eye irritation	Mycotrol WP9616b (BotaniGard 22 WP)	Rabbit	Non irritant	--	Johnson, W. D. (1997) TOX2006-877
Skin sensitisation		Not submitted		R 43 *)	

*) This classification is assigned in the absence of a sensitisation test in accordance with current guidelines (2001/36/EC and 2005/25/EC) and also proposed by the notifier. A study is currently underway, results are announced for 2006.

BotaniGard 22 WP is of low acute toxicity following the oral route of exposure. The rat oral LD_{50} is higher than 5000 mg/kg bw. Based on the negligible acute dermal toxicity of the active substance and the co-formulants and since the micro-organism does not pass through the skin barrier an acute dermal study was not carried out for reasons of vertebrate protection. The conditions for reporting the inhalation toxicity of the plant protection product according to Directive 94/79/EC, amending Directive 91/414/EEC are not fulfilled and hence testing for inhalation toxicity is not relevant and has not been conducted. Even if the inhalation toxicity of the active ingredient in the rat is taken into consideration a low acute inhalation toxicity is predicted. In acute irritation tests with BotaniGard 22 WP the formulation was not irritant to the skin or to eyes in rabbits. A classification with Xi; R 43 is assigned in the absence of a sensitisation test according to directives 2001/36/EC and 2005/25/EC. A dermal sensitisation study was announced for 2006.

In addition, transient local reactions have been observed following intratracheal challenge of rats with *Beauveria bassiana* strain GHA as well as subsequently to intramuscular injection to rats or inhalative exposure of Guinea pigs. These observations are in line with the assumption of an immunological reaction to the micro-organism. Based on these findings, and taking into account the evidence of allergenicity in humans (see section B.6.1.1), inhalation seems to be more critical than dermal contact and, therefore, classification and labelling as a potential inhalatory and skin allergen (Xn, R42/43) is required for *Beauveria bassiana* strain GHA (see B.6.1.2). Hence, according to the Directive 1999/45/EC classification of BotaniGard 22 WP with Xn, R42/43 is necessary, too.

In accordance with Directives 67/548/EEC and 1999/45/EC and on the basis of the results from acute toxicity testing, the following classification/labelling requirements are derived for BotaniGard 22 WP:

Hazard symbol(s)	Xn	
Indications of danger	Harmful	
Risk phrase(s)	R 42	May cause sensitisation by inhalation
	R 43	May cause sensitisation by skin contact

B.6.4.1.1 Acute oral toxicity

Reference:	IIIM 7.1.1/01
Report:	Johnson, W. D. (1997) Acute oral toxicity study of Mycotrol WP9616b in rats (Limit test) (Project No. L08608, Study No.15) TOX2006-875
Guidelines:	US EPA Guideline 81-1 (comparable to the recommended EU guideline from Directive 2004/73/EEC, Method B.1)
Deviations:	None
GLP:	Yes (self-certified in accordance with US 40 CFR 160)
Acceptability:	The study is considered to be acceptable.

Material and methods:

The formulated product Mycotrol WP9616b [= BotaniGard 22 WP] was fed as 50 % corn oil suspension to 5 male and 5 female Sprague-Dawley (Crl:CD BR Strain) fasted rats by oral gavage at a single, limit dose equivalent to 5 g BotaniGard 22 WP/kg bodyweight (weight at dosing 220 - 241 g males; 160 - 171 g females). The rats were housed individually in stainless steel cages between 22.0 and 24.5 ºC and at relative humidity between 42 and 63 %. Animals were observed for a 14-day period after administration. Body weights were recorded at weekly intervals. At the end of the study the rats were sacrificed by CO_2 asphyxiation and subjected to gross necropsy.

Findings:

There were no mortalities during the study in any group. The only overt clinical symptoms noted during the study were redness around the nose fur in 2 males on day 1. The following day all animals were normal. Weight gains were normal, within expected limits and nothing remarkable was observed during the remainder of this study. No gross necropsy findings were observed.

The findings are summarised in Table B.6.4-3.

Table B.6.4-3 Acute oral toxicity of BotaniGard 22 WP (dose, mortality/animals treated)

Dose (mg/kg bw)	Males	Females
5000	0/5	0/5

Conclusion:

There were no mortalities in rats exposed to a test concentration of 5000 mg/kg BotaniGard 22 WP by oral application. Therefore the oral LD_{50} was estimated to be greater than 5000 mg/kg bw. In accordance with the provisions of Council Directive 67/548/EEC, BotaniGard 22 WP does not warrant classification on the basis of its acute oral toxicity.

B.6.4.1.2 Acute inhalation toxicity

Justification for non-submission as given by the notifier:

No inhalation studies have been conducted on BotaniGard 22 WP.

Rationale (91/414/EEC, Annex III, 7.1.3 Inhalation):

The inhalation toxicity of the plant protection product must be reported where it is:
- A gas or liquefied gas,
- Is a smoke-generating formulation or fumigant,
- Is a vapour releasing preparation,
- Is used with fogging equipment,
- Is an aerosol,
- Contains an active substance with a vapour pressure > 1 x 10^{-2} Pa *and* is to be used in enclosed spaces such as warehouses or glasshouses,
- Is a powder containing a significant proportion of particles of diameter < 50 µm (> 1 % on a weight basis),
- Is to be applied from aircraft in cases where inhalation exposure is relevant,
- Is to be applied in a manner which generates a significant proportion of particles or droplets of diameter < 50 µm (> 1 % on a weight basis).

This formulation does not fall into any of the above categories and hence testing for inhalation toxicity is not relevant and has not been conducted.

Remarks by the RMS

Following intratracheal administration to rats, *Beauveria bassiana* strain GHA was not infective. The micro-organism also proved to be not pathogenic and not toxic in terms of inducing an ongoing systemic disease process. The acute intratracheal LD_{50} was > 1.01×10^8 CFU for males and >1.05×10^8 CFU for female rats. However, there was evidence of a local adverse reaction to treatment in the lungs (see B.6.1.2.2.2). No classification is needed. The justification for non-submission will be accepted.

B.6.4.1.3 Acute percutaneous toxicity

Justification for non-submission as given by the notifier:

A study conducted with the active substance *Beauveria bassiana* strain GHA indicated that the LD_{50} was >2000 mg/kg bw. The micro-organism does not pass through the skin barrier, and the co-formulants are not classified as toxic. Thus in the interests of reduction of vertebrate mammalian toxicity studies, this study was not conducted. The MPCP, BotaniGard 22 WP, is not considered to be "Harmful" or "Toxic" by the dermal route.

Remarks by the RMS

The acute dermal LD_{50} of *Beauveria bassiana* strain GHA in rabbits was > 1.6×10^{11} CFU/animal. No evidence of infectivity, pathogenicity or systemic toxicity was obtained. However, slight dermal irritation was observed (see B.6.2.1). No classification is needed. The justification for non-submission will be accepted.

B.6.4.2 Additional acute toxicity studies - The preparation

B.6.4.2.1 Skin irritation

Reference:	IIIM 7.1.4/01
Report:	Findlay, J. F. (1999) Acute dermal irritation study of Mycotrol Botani Gard 22 WP in rabbits (Project No. 8608 SN32) TOX2006-876
Guidelines:	US EPA OPPTS No. 870.2500, OECD Guideline 404 (comparable to the recommended EU guideline from Directive 2004/73/EEC, Method B.4)
Deviations:	None
GLP:	Yes (self-certified in accordance with US 40 CFR 160)
Acceptability:	The study is considered to be acceptable.

Material and methods:

Mycotrol BotaniGard 22 WP, moistened with purified water, was applied undiluted to the shaven backs of six New Zealand White rabbits (3 male, 3 female) at a dose of 0.5 g each and covered with a 2.5 x 2.5 cm gauze patch, secured with porous tape. After 4-h exposure the treatment site was cleansed. The rabbits were housed individually in suspended stainless steel cages between 21 and 23 ºC and at relative humidity between 28 and 38 %. The weight range for rabbits used in this study was 2.71 - 3.04 kg at treatment initiation.

The test sites were examined for signs of dermal irritation such as oedema, erythema and/or eschar formation, and for corrosivity such as ulceration and/or necrosis, at 30 - 60 minutes, 24, 48 and 72 hours after removal of the wrappings. All findings were evaluated according to the criteria and scores of Draize.

Findings:

No deaths occurred during the study. All rabbits gained weight during the study. Very slight to well-defined erythema was noted in all animals at 1 hour after removal of the wrappings. Very slight oedema was seen in 2 animals at the 24-h period, and slight erythema persisted in 4 animals until the 48-h period. All animals were completely recovered from all signs of dermal irritation by the 72-h scoring period. Individual mean animal mean irritation scores ranged from 0.38 to 0.88, with the Primary Irritation Index for BotaniGard 22 WP at 0.53 [mild or slight irritation].

The findings are summarised in Table B.6.4-4.

Table B.6.4-4: Summary of Dermal Irritation Scores of BotaniGard 22 WP

Time interval	Skin Reaction	Rabbit Number					
		066	067	068	063	064	065
30-60 minutes	Oedema	1	0	0	0	1	1
	Erythema/eschar formation	2	1	2	1	1	1
24 hours	Oedema	1	0	0	1	0	0
	Erythema/eschar formation	2	1	1	1	2	1
48 hours	Oedema	0	0	0	0	0	0
	Erythema/eschar formation	1	1	0	1	1	0
72 hours	Oedema	0	0	0	0	0	0
	Erythema/eschar formation	0	0	0	0	0	0
Mean score		0.88	0.38	0.38	0.50	0.63	0.38

Conclusion:

On the basis of the degree of skin reaction observed (mean erythema and oedema scores at 24 to 72 hours after removal of the test article < 2.0) classification as a skin irritant according to EC-criteria is not required.

B.6.4.2.2 Eye irritation

Reference:	IIIM 7.1.5/01
Report:	Johnson, W. D. (1997) Primary eye irritation study of Mycotrol WP9616b in rabbits (Project No. L08608, Study No. 7) TOX2006-877
Guidelines:	US EPA Guideline 152A-14 (comparable to the recommended EU guideline from Directive 2004/73/EEC, Method B.5)
Deviations:	None
GLP:	Yes (self-certified in accordance with US 40 CFR 160)
Acceptability:	The study is considered to be acceptable.

Material and methods:

Mycotrol WP9616b [= BotaniGard 22 WP] was administered undiluted at a dose of 0.1 g to the right eye of each of six New Zealand White rabbits. The untreated left eye of each rabbit served as a control for comparison purposes. Each rabbit received a dose of 4.61×10^9 viable spores (conidia). The right eye of each rabbit was rinsed with water 24 hours after instillation of the test substance. The rabbits were housed individually in suspended stainless steel cages between 24 to 25 ºC, and at relative humidity between 24 and 63 %. The weight range for rabbits used in this study was 2.56 - 2.96 kg at dosing.

All eyes were examined pre-treatment to ensure that there were no corneal lesions before treatment. The treated eye of each rabbit was scored for irritation at 1, 24, 48 and 72 hours and at 4, 7, 10 and 14 days following the administration of the test substance. The treated and control eyes of the rabbits were examined at the intervals given above using fluorescein (not for 1 hour interval) and UV light. The cornea was examined for the area, presence and degree of opacity; the iris for deepened rugae (folds), congestion, swelling, circumcorneal hyperaemia, and reaction to light; and the conjunctiva for redness, chemosis and discharge.

Findings:

There were no mortalities in the study. The highest 'Mean Eye Irritation Score (24 - 72 h) observed during the study was 1.39 (Chemosis). Except for pannus formation in one rabbit, signs of ocular irritation were no longer seen in any rabbit at the 10-day scoring interval. The treated eyes of all animals appeared to be normal at 14 days post-treatment.

The individual findings of each rabbit are specified in Table B.6.4-5.

Table B.6.4-5: Individual Eye Irritation Scores of Mycotrol WP9616b : (BotaniGard 22 WP)

Animal Number	1 hour		24 hours		48 hours		72 hours		4 days		7 days		10 days		14 days	
Cornea (A=Density of Opacity, B=Area of Opacity)																
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
628	0	0	2	4	2	4	2	4	2	3	1	1	0	0*)	0	0
629	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
630	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	0
631	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0
632	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
633	0	0	2	2	0	0	0	0	0	0	0	0	0	0	0	0
Mean scores 24-72 h			0.67 (A)													
Iris																
628	1		1		1		1		1		0		0		0	
629	0		1		0		0		0		0		0		0	
630	0		1		0		0		0		0		0		0	
631	1		1		0		0		0		0		0		0	
632	1		1		0		0		0		0		0		0	
633	0		1		0		0		0		0		0		0	
Mean scores 24-72 h			0.44													
Conjunctiva (A=Erythema, B=Chemosis, C=Discharge)																
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
628	2	2	2	3	3	2	2	2	1	2	2	1	1	1	0	0	0	0	0	0	0	0	0	0
629	1	1	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
630	1	1	2	2	3	3	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
631	1	2	2	1	2	2	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
632	2	2	3	2	2	2	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
633	1	2	3	1	4	3	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mean scores 24-72 h				1.06 (A) 1.39 (B) 0.83 (C)																				

*) Pannus formation observed

Conclusion:

On the basis of reactions observed (mean eye irritation scores at 24 to 72 hours after instillation of the test material) classification as an eye irritant according to EC-criteria is not required.

B.6.4.2.3 Skin sensitisation

Reference: IIIM 7.1.6

Report: Arcelin, G. (2006), BOTANIGARD® 22WP: Contact Hypersensitivity in Albino Guinea Pigs, Bühler Test
RCC Ltd, Toxicology, CH-4452 Itingen / Switzerland
Unpublished RCC Study Number A38575, June 19, 2006
1679375

Guidelines: OECD 406 (1992)
EC method B.6 - Buehler-Test

Deviations: None

GLP: Yes

Acceptability: The study is considered to be acceptable.

Material and methods:

The purpose of this skin sensitising study was to assess the possible allergenic potential of BotaniGard 22 WP when administered topically to albino Dunkin Hartley guinea pigs.

For this purpose the "Buehler Test" modified by Ritz, H.L. and Bühler, E.V. (1980)[1] was used. Twenty male animals of the test group were treated topically with BotaniGard 22 WP at 50 % in purified water once a week for a 3-week induction phase. The test item concentration of 50 % in purified water was considered to be the most qualified to assure an optimum technical application procedure. Two weeks after the final induction application the animals were challenged with the test item concentration of 3 % in purified water.

The ten animals of the control group were not treated during the induction. They were treated once at challenge with BotaniGard 22 WP at 3 % in purified water.

Findings:

The highest tested non-irritating concentration of BotaniGard 22 WP used for challenge was 3 % in purified water. The incidence of positive erythema reactions after topical challenge is described in Table B.6.4-6.

[1] Current Concepts Cutaneous Toxicity, ed. Drill, V.A. and Lazar, T. (Academic Press, 1980) pp. 25-40: Planning, Conduct and Interpretation of Guinea Pig Sensitization Patch Tests.

Table B.6.4-6: BotaniGard 22 WP - Responses to challenge applications

Erythema Score	Test Group 20 animals		Control Group 10 animals	
	24 hrs	48 hrs	24 hrs	48 hrs
0	4	1	7	8
1	14	16	2	1
2	2	3	0	0
3	0	0	0	0
No. with grades ≥ 1	16	19	2	1
No. tested	20	20	9	9
Incidence *)	**19/20**		**2/9** ***)	
Severity **)	**0.9 - 1.1**		**0.11 - 0.22**	

*) Number of animals showing a response of grade 1 or greater at either 24- or 48-hour reading out of the total animals.

**) Total sum of 24- and 48-hour response readings divided by the number of animals exposed (maximum of 3).

***) One animal of the control group died on test day 29 during the challenge application. At necropsy, no macroscopic findings were noted. The cause of death could not be established. The death was considered to be spontaneous and treatment unrelated.

The sensitivity and reliability of the experimental technique employed was assessed by use of Alpha-Hexylcinnamaldehyde which is recommended by the OECD 406 Guidelines and is known to have moderate skin sensitisation properties in the guinea pig strain.

Conclusions:

In this study 95 % and 22 % of the animals of the test and control group, respectively were observed with skin reactions after challenge treatment performed with the highest tested non-irritating concentration of BotaniGard 22 WP at 3 % in purified water.

The nature of the reactions seen in the control group (fading at the 48-hour reading in one of the two positive cases previously observed at the 24-hour reading) suggested they might in fact due to skin irritation. Since, these must be irritant effects, the skin reactions cast doubt on the nature of the reactions apparent in the test animals particularly if they are of same grade. However, the absence of skin reaction fading in the test group between the 24- and 48-hour reading and a tendency of the skin reactions to increase in incidence and severity (booster effect) at the later time point confirmed the allergic nature of the reactions in the test group.

Based on the above mentioned findings in a non-adjuvant sensitisation test in guinea pigs and in accordance to Commission Directive 2001/59/EC, BotaniGard 22 WP applied at a concentration of 3 % in purified water has to be classified and labelled as a skin sensitiser.

B.6.4.3 Data on exposure - The preparation (OECD IIIM 7.2)

BotaniGard 22 WP is a wettable powder formulation (WP) containing *Beauveria bassiana* strain GHA (pure 220 g/kg or 4.4 x 10^{13} CFU/kg).

The dossier contains data and information to support a limited range of representative uses of the active substance for which it is intended to demonstrate that, for one preparation, the requirements of Commission Directive 91/414/EEC and Council Directive 2001/36/EC and

additional SANCO documents, as specified in Commission Regulation (EC) No. 1112/2002/EC are fulfilled. These uses are intended to include the major commercial applications. The intended uses - which will be applied with a knapsack sprayer - are:

- ornamentals in greenhouses
- vegetables in greenhouses

The exposure occurs via different routes (dermal and inhalation). The acute dermal study with strain GHA revealed evidence of slight irritation but proved the absence of infectivity, pathogenicity and toxicity when applied by this route (see B.6.2.4). Therefore, a systemic dose from dermal contact is not considered to be of toxicological relevance as no systemic effects are to be expected after topical exposure to *Beauveria bassiana* strain GHA.

However, clearly treatment-related systemic and local effects have occurred in the acute study with intratracheal administration to rats (Barbera, 1993, TOX2006-872). The effects were not fully reversible until termination. Since only one dose level was used in this study, a threshold level for adverse effects or a NOAEL could not be determined. Of course, intratracheal application is highly artificial and does not completely reflects practical exposure but rather worst-case conditions. Nonetheless, an inhalative risk cannot be excluded and the lung seems to be particularly sensitive. This assumption is further substantiated by data from the open literature (Fromtling *et al.*, 1979, TOX2007-472; Melnikova and Murza, 1980, TOX2007-478; Song, 1989, TOX2007-481). There is evidence of allergenicity by inhalation in laboratory animals and humans and (weaker) indications of allergic skin reactions in humans.

The notifier has proposed a tolerable exposure level of 50 mg/kg bw/day, based on the acute oral LD_{50} of > 5000 mg/kg bw/day and using an assessment factor of 100.

Because of different effects due to dermal and inhalation exposure, dermal and inhalation exposure scenarios should be taken into account for the risk assessment. For *Beauveria bassiana* strain GHA, the inhalation exposure seems to be the relevant route. Since there is neither a NOAEC derived by a subacute or chronic inhalation study nor a relevant NOAEL of a corresponding oral or dermal study available, with which estimated exposure data could be compared, a quantitative risk assessment cannot be carried out. In order to gain insight into potential acute risk to operators or workers at all, estimated exposure levels could be discussed in relation to the LOAEL (i.e. 5.7 mg as/kg bw for males and 6.7 mg as/kg bw for females, respectively) of the above cited acute inhalative study or to the LD_{50} of the acute dermal toxicity study.

In the open literature, *Beauveria bassiana* is described to produce a range of metabolites. However, there is only an analytical method for beauvericin available. Hence, it is proposed to use the beauvericin content as an indicator for the presence of the other metabolites. Estimation of operator and worker exposure with this metabolite was performed with the max. content of 50 ppm beauvericin in the technical material. In order to consider all metabolites sufficiently it is proposed to specify beauvericin at a maximum level of the technically feasible 5 ppm (see B.6.2.4).

B.6.4.3.1 Estimation of Operator exposure

Operator exposure estimates were calculated using the German model and the Greenhouse model (ECON, 1996):

– Uniform Principles for Safeguarding the Health of Applicators of Plant Protection Products (Uniform Principles for Operator Protections); Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirschaft, Berlin-Dahlem, no. 277, 1992;

– Mich, G. (1996); Operator Exposure in Greenhouses During Practical Use of Plant Protection Products, ECON Forschungs- und Bewertungskonzepte für Umwelt und Gesundheitssicherheit GmbH, Project EF 94-02-03.

The operator exposure has been calculated without PPE

The following assumptions are made for the estimation of operator exposure:

Application rate:	max. 0.121 kg as/ha
Work rate:	1 ha/day
Formulation type:	WP
Body weight of an operator:	70 kg

Using the input parameters and the scheme of the calculation model (appendix 1), the estimated operator exposure can be calculated for mixing/loading (m/l) and application (appl.). The results for the estimated dermal and inhalation exposures are given in Table B.6.4-7:

Table B.6.4-7: Estimated operator exposure using the German model and the Greenhouse model (high crops)

Exposure route and type of work	Estimated operator exposure Greenhouse application (high crop) (mg/person/d)
	without PPE
Dermal exposure	
Mixing/loading	6.05
Application	11.76
Total, dermal	17.81
(mg/kg bw)	(0.254)
Inhalation exposure	
Mixing/loading	0.097
Application	0.013
Total, inhalation	0.110
(mg/kg bw)	(0.002)

Using the German model and the Greenhouse model (high crop), the estimated inhalation exposure is calculated to be max. 0.11 mg/person/d (0.002 mg/kg bw). The estimated dermal exposure without PPE is calculated to be max. 17.81 mg/person/d (0.254 mg/kg bw).

Taking into account the possible production of the metabolite beauvericin at a level of max. 50 mg/kg the estimated exposure to beauvericin without PPE would be max. 0.013 µg/kg bw.

Comparison of estimated and tolerable exposure

Due to the lack of a medium term tolerable inhalation exposure level, a comparison with the estimated exposure is not possible.
As a very rough estimate calculated inhalation exposure values are compared with the LOAEL derived from an acute inhalation toxicity study (see above). In the case of greenhouse applications the estimated inhalation exposure of max. 0.002 mg/kg bw is considerably below the observed LOAEL of 5.7 mg as/kg bw of males (worst case). Since BotaniGard 22 WP has to be classified and labelled with R 42/43 (c.f. B.6.5) the use of respiratory protection equipment (half-mask) during handling the product is strongly recommended. This would reduce the possible inhalative exposure, thus enhancing the margin between the estimated exposure and the acute LOAEL available.

Although it can be assumed that systemic exposure by the dermal route is of minor relevance the estimated potential dermal exposure can be set in relation to the dermal LD_{50} only, which has been established to be > 470 mg as/kg bw in male rabbits (worst case). Estimated potential dermal exposure value during greenhouse application (0.25 mg/kg bw) is well below this value.

In addition, it is concluded that the operator exposure to the metabolite beauvericin (estimated max. 0.013 µg/kg bw) is unlikely to exceed the proposed conservative threshold of toxicological concern (TTC) of 0.02 µg/kg bw/d (see B.6.2.4).

Taken all together, it can be concluded that operators would not be at acute risk during mixing/loading and application of BotaniGard 22 WP. Although situations of repeated exposure at subacute dose levels are not covered by the above considerations, it can be assumed that the estimated operator exposure is acceptable provided appropriate RPE is worn. In addition, precautionary the use of suitable gloves as well as wearing suitable protective clothing is considered necessary to minimise the risk of skin sensitisation because of the skin sensitising properties of BotaniGard 22 WP. Furthermore operators should know that eye infections were observed after mechanical injury of the cornea (see B.6.1.1.4).

B.6.4.3.2 Bystander exposure

Bystander exposure is not relevant for the intended uses (greenhouse).

B.6.4.3.3 Worker exposure

BotaniGard 22 WP will be applied on ornamentals and vegetables in greenhouses.

The greatest potential for worker exposure following re-entry will be skin contamination. Inhalation exposure, on the other hand, is generally confined to a brief period after application, while the product is drying. A worst case estimation of worker exposure is presented below.

The level of exposure will depend largely on the dislodgeable foliar residue (DFR), the length of time the residue remains on the surface of the crops and the degree of contact with the foliage. In order to consider such a situation an estimation is based on the model as developed by the German BBA (Biologische Bundesanstalt) [Hoernicke E. et al.; 1998; Hinweise in der Gebrauchsanleitung zum Schutz von Personen bei Nachfolgearbeiten in mit Pflanzenschutzmitteln behandelten Kulturen (worker re-entry; Nachrichtenbl. Deut. Pflanzenschutzd. 50, Berlin].

The re-entry/dermal exposure (D) is calculated by the formula:

Dermal exposure = DFR x TF x A x (P) x R

Assumptions of the re-entry model	
DFR (dislodgeable foliar residues):	1 µg/cm^2/kg as *)
TF (transfer factor):	5000 cm^2/person x h **)
A (work rate per day):	8 h/d
R (application rate):	0.121 kg as/ha

Dermal exposure
D = 1 µg/cm^2/kg as *) x 5000 cm^2/person x h **) x 8 h/d x 0.121 kg as/ha
D = 4.84 mg/person/d (dermal exposure, not wearing PPE)

*) assuming the use of footwear and long trousers
**) worst case (acc. to EUROPOEM II, 2002)

Dermal exposure [mg/kg bw/d] = D [mg/person/d] x AF / BW [kg/person]

Assumptions / derived values	
AF (dermal absorption rate/absorption factor):	100 %
BW (body weight):	60 kg

Resulting exposure
D = 4.84 mg/person/d x 100 % / 60 kg bw/person
D = 0.081 mg/kg bw/d (dermal exposure, not wearing PPE)

Based on the worst case assumptions, the estimated worst-case worker exposure would be 0.081 mg/kg bw/d for a person not wearing PPE.

Taking into account the possible production of the metabolite beauvericin at a level of max. 50 mg/kg the estimated exposure to beauvericin without PPE would be max. 0.004 µg/kg bw.

Comparison of estimated and tolerable exposure

The acute dermal LD_{50} of *Beauveria bassiana* strain GHA in rabbits was > 1.6x 10^{11} CFU/animal (corresponding to about 470 or 630 mg as/kg bw). Slight dermal irritation was observed, however, no evidence of infectivity, pathogenicity or systemic toxicity was obtained (see B.6.2.1). Therefore, after the spray solution has dried, the estimated exposure to *Beauveria bassiana* strain GHA during re-entry operations does not exceed acceptable values, even if no PPE is worn. Nevertheless, precautionary the use of suitable gloves as well as

wearing suitable protective clothing is considered necessary to minimise the risk of skin sensitisation because of the skin sensitising properties of BotaniGard 22 WP.

Appendix 1

Operator exposure for *Beauveria bassiana* strain GHA in greenhouses (high crop, geom. mean) based on German model (mix./load) and Mich (1996) (appl.)

Assumptions and input parameters considered for the estimation of the operator exposure:

Formulation type:	WP	**DM(H)**	= 50 mg/person x kg as
Application rate:	0.121 kg *Beauveria bassiana* strain GHA/ha	**DA(H)**	= 13.1884 mg/person x kg as
Area treated per day:	1 ha	**DA(C)**	= 1.56194 mg/person x kg as
Dermal absorption rate (m/l):	100 %	**DA(B)**	= 82.4751 mg/person x kg as
Dermal absorption rate (appl.):	100 %	**IM**	= 0.8 mg/person x kg as
Inhalation absorption rate:	100 %	**IA**	= 0.10841 mg/person x kg as
Body weight:	70 kg		

Route of exposure			**without PPE**
Dermal exposure			
Dermal (mix.load.)			
exposure (hands):	DM(H)	=	50 x 0.121 x 1
		=	6.05 mg/pers. x d
Dermal (application)			
exposure (hands)	DA(H)	=	13.1884 x 0.121 x 1
		=	1.60 mg/pers. x d
exposure (head)	DA(C)	=	1.56194 x 0.121 x 1
		=	0.19 mg/pers. x d
exposure (body)	DA(B)	=	82.4751 x 0.121 x 1
		=	9.89 mg/pers. x d
Dermal (application)	DA	=	11.76 mg/pers. x d
Total dermal exposure	D	**=**	**17.81 mg/pers. x d**
		=	**0.2545 mg/kg bw x d**
Inhalation exposure			
Inhalation (mix.load.)			
	IM	=	0.8 x 0,121 x 1
		=	0.097 mg/pers. x d
Inhalation (application)			
	IA	=	0.10841 x 0.121 x 1
		=	0.013 mg/pers. x d
Total inhalation exposure	I	**=**	**0.1099 mg/pers. x d**
		=	**0.0016 mg/kg bw x d**

B.6.4.4 Available toxicological data relating to non-active substances - The preparation (OECD IIIM 7.4)

BotaniGard 22 WP is formulated as a wettable powder containing nominal 220 g/kg *Beauveria bassiana* strain GHA. Besides its active ingredient, the preparation contains some co-formulants partly with irritating properties. The respective information is given in the safety data sheets of the notifier.

Possible acute toxic properties (oral) and acute skin and eye irritating properties of co-formulants are covered by the studies with the preparation. Other information provided on the non-active substances present in BotaniGard 22 WP indicate no further specific toxicological properties of concern.

B.6.4.5 Supplementary studies for combinations of plant protection products (OECD IIIM 7.5)

No studies in combination with other plant protection products are necessary as its use in combination with other plant protection products and/or with adjuvants as a tank mix is not proposed.

B.6.5 Summary and evaluation of health effects - The preparation (OECD IIIM 7.6)

BotaniGard 22 WP is a wettable powder formulation (WP) containing *Beauveria bassiana* strain GHA (pure 220 g/kg or 4.4 x 10^{13} CFU/kg). The preparation is intended for use on ornamentals and vegetables in greenhouses.

A summary of the results of the acute toxicity studies including irritancy and skin sensitisation can be found in Table B.6.5-1.

Table B.6.5-1: Summary of acute toxicity of BotaniGard 22 WP

Parameter	Test substance	Species	Results	Danger symbol, R-phrase	Reference
Acute oral LD_{50}	Mycotrol WP9616b (BotaniGard 22 WP)	Rat	> 5000 mg/kg bw	--	Johnson, W. D. (1997) TOX2006-875
Acute inhalative LD_{50}		Not submitted (*Beauveria bassiana* strain GHA: Intratracheal study, LC_{50} rat > 1 x 10^8 CFU/animal; evidence of some inhalation toxicity, sensitisation by inhalation cannot be excluded)		--	See B.6.3
Acute dermal LD_{50}		Not submitted (*Beauveria bassiana* strain GHA LD_{50} rabbits > 1.6x 10^{11} CFU/animal, no systemic effects but signs of slight but persisting local irritation)		--	See B.6.3
Acute skin irritation	Mycotrol (BotaniGard 22 WP)	Rabbit	Non irritant	--	Findlay, J. (1999) TOX2006-876
Acute eye irritation	Mycotrol WP9616b (BotaniGard 22 WP)	Rabbit	Non irritant	--	Johnson, W. D. (1997) TOX2006-877
Skin sensitisation (Buehler test with 3 inductions)	BotaniGard 22 WP	Guinea pig	Sensitising	R 43	Arcelin, G. (2006) ASB2007-4107

BotaniGard 22 WP is of low acute toxicity following the oral route of exposure. The rat oral LD_{50} is higher than 5000 mg/kg bw. Based on the negligible acute dermal toxicity of the active substance and the co-formulants and since the micro-organism does not pass through the skin barrier an acute dermal study was not carried out for reasons of vertebrate protection. The conditions for reporting the inhalation toxicity of the plant protection product according to Directive 94/79/EC, amending Directive 91/414/EEC are not fulfilled and hence testing for inhalation toxicity is not relevant and has not been conducted. Even if the inhalation toxicity of the active substance in the rat is taken into consideration a low acute inhalation toxicity is predicted. In acute irritation tests with BotaniGard 22 WP the formulation was not irritant to the skin or to eyes in rabbits. Based on the findings in a non-adjuvant sensitisation test in guinea pigs and in accordance to Commission Directive 2001/59/EC, BotaniGard 22 WP has to be classified and labelled as a skin sensitiser.

In addition, transient local reactions have been observed following intratracheal challenge of rats with *Beauveria bassiana* strain GHA as well as subsequently to intramuscular injection to rats or inhalative exposure of guinea pigs. There is also evidence of allergenicity by inhalation in humans and (weaker) indications of allergic skin reactions in humans. These observations are in line with the assumption of an immunological reaction to the micro-organism. Based on these findings inhalation seems to be more critical than dermal contact.Classification and labelling as a potential inhalatory and skin allergen (Xn, R 42/43) is required for *Beauveria bassiana* strain GHA (see B.6.3). Hence, according to the Directive 1999/45/EC classification

of BotaniGard 22 WP with Xn, R42 is necessary, too. Furthermore, published findings suggest a possible affinity of *Beauveria bassiana* to ocular tissues when the cornea once was penetrated (see B.6.1.1.4).

In accordance with Directives 67/548/EEC and 1999/45/EC and on the basis of the results from acute toxicity testing, the following classification/labelling requirements are derived for BotaniGard 22 WP:

Hazard symbol(s)	Xn	
Indications of danger	Harmful	
Risk phrase(s)	R 42	May cause sensitisation by inhalation
	R 43	May cause sensitisation by skin contact

In addition, operators should be informed in a suitable way that eye infections were observed after mechanical injury of the cornea.

B.6.6 References relied on

Annex point/ reference number	Author(s)	Year	Title source (where different from company) report no. GLP or GEP status (where relevant), published or not BVL registration number	Data protection claimed Y/N	Owner[2]
KIIM 5.1 (OECD)	Fuguet, R. and Vey, A.	2004	Comparative analysis of the production of insecticidal and melanizing macromolecules by strains of Beauveria spp.: in vivo studies. J Invertebr. Pathol. 85, 152-167 GLP: O, published: Y 1300974 / TOX2007-483	N	LIT
KIIM 5.1 KIIM 5.2 (OECD)	Beaumont, F., Kauffmann, H.F., Demonchy, J.G.R., Sluiter, H.J., Devries, K.	1985	Volumetric aerobiological Survey of Conidial Fungi in the Northeast Netherlands. 1. Comparison of Aerobiological data and Skin-Tests with Mold Extracts in an Asthmatic Population. Allergy 40, 181-186 GLP: O, published: Y 1300975 / TOX2007-470	N	LIT
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KIIM 5.2 (OECD)	Bradley, C.	1993	Discussion of formation of unintentional ingredients. GLP: O, published: N 1300905 / TOX2006-898	Y	LAM
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KIIM 5.3.1 (OECD) KIIIM1 7.1.6	Arcelin, G.	2006	Botanigard 22WP: Contact hypersensitivity in albino guinea pigs, Bühler test A38575 GLP: Y, published: N 1679375 /	Y	MEU
KIIM 5.3.2 (OECD)	Barbera, P. W.;	1993	Toxicity/pathogenicity testing of Beauveria bassiana strain GHA Following acute oral challenge in rats L08433 SN6 GLP: Y, published: N 1300907 / TOX2006-871	Y	LAM
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KIIM 5.5 (OECD)	Johnson, W. D.	1993	Acute dermal toxicity study of Beauvaria bassiana GHA in rabbits L08433 SN3 GLP: Y, published: N 1300909 / TOX2006-874	Y	LAM
KIIIM1 7.1.1 (OECD)	Johnson, W. D.	1997	Acute oral toxicity study of Mycotrol WP96116b in rats (Limit test) L08608 No15 GLP: Y, published: N 1301039 / TOX2006-875	Y	LAM
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LIT	Literature
MEU	Mycotech Europe Ltd.