Renewal Assessment Report

Lecanicillium muscarium Ve6

Volume 3MA – B.6 Effects on human health

January 2018

Rapporteur Member State: The Netherlands
Co-Rapporteur Member State: France

Version history

When	What
January 2018	Initial RAR

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B Summary of the data and information

B.6 Effects on human health

Note to reader:
Information from the original DAR and/or addenda to the DAR is highlighted grey.

The company Koppert B.V. is submitting a dossier for the re-approval of the microorganism *Lecanicillium muscarium* Ve6 (19-97), further referred to as *Lecanicillium muscarium* Ve6, as an active ingredient under Regulation (EC) 1107/2009.

The Microbial Pest Control Agent *Lecanicillium muscarium* Ve6 (formerly *Verticillium lecanii* Ve6) was included in Annex I of Directive 91/414/EEC on 1 May 2009 pursuant to Article 24b of the Regulation (EC) No 2229/2004, (Commission Directive 2008/113/EC) and then approved according to the Commission Implementing Regulation (EU) No 540/2011 of 25 May 2011, implementing Regulation (EC) No 1107/2009 of the European Parliament. *L. muscarium* Ve6 was notified and defended by Koppert Beheer B.V. (Koppert B.V. is a 100% daughter company of Koppert Beheer B.V.). The active ingredient has been evaluated in The Netherlands according to Uniform Principles. The representative formulated product for the initial evaluation was the product MYCOTAL, containing 1.0×10^{10} spores/g.

The microorganism has been previously classified as *Verticillium muscarium.* The strain has been reclassified in 2001 as *Lecanicillium muscarium*, based on molecular analysis as RFLP and ITS sequence analysis by Zare & Gams. The taxonomy change was already considered for the peer review of the pesticide risk assessment of the active substance by EFSA[1].

Here the data is presented that were previously evaluated by RMS The Netherlands in the DAR (June 2007) and DAR addenda (June 2009, October 2009), as well as new data and information based on literature searches and studies. Previously submitted information (consolidated from DAR and addenda) is highlighted in grey, and information on the original DAR Points and the respective EU Points is complemented where necessary.

B.6.1 Tier I

B.6.1.1 Basic information

New data 2016

[1] European Food Safety Authority; Conclusion on the peer review of the pesticide risk assessment of the active substance *Lecanicillium muscarium* strain Ve6, notified as *Verticillium lecanii*. EFSA Journal 2010; 8(1):1446. [45 pp.]. doi:10.2903/j.efsa.2010.1446. Available online: www.efsa.europa.eu

A literature search was conducted to identify relevant peer reviewed open literature reporting on human toxicity of *Lecanicillium muscarium* (Pfau 2016). The literature research was performed according the EFSA (2011) guidance document. The search was based on the genus level of *Lecanicillium* due to only few hits on the species level. The search considered also the *L. muscarium* Ve6 formulated product MYCOTAL, as well as references reporting on *Verticillium lecanii* since the taxonomy of *Lecanicillium muscarium* has been changed in the past years. Therefore, relevant literature may not consider the changes in taxonomy and the current designations of *Lecanicillium muscarium*. The report summarises an evaluation of a literature search performed according to the EFSA document. The search strategy was based on relevance criteria and reliability criteria (see criteria). The databases selected are MEDLINE; BIOSIS, CAB, and SCISEARCH. The search terms used were *Lecanicillium* or Mycotal, or *Verticillium lecanii* and 2006-2016 and human and (rat or mouse or guinea pig or dog or primate or vitro). So, the search included typical terms targeting references on toxicity on mammals. After rapid assessment 8 references of the 264 were identified as being potentially relevant and subjected to detailed assessment of full-text documents. From of the total of 264 hits (after removal of duplicates) only four articles were considered relevant for the data point: Madsen 2007 (B.6 mp), Neal 2012 (B.6 ma), Baelum 2012 (B.6 ma) and Kouvelis 2011 (B.6 ma). Four non-relevant articles are also identified: one review article with no new data and three articles about isolated substances as cyclic lipodepsipeptides verlamelin A and B, lecanindoles and verticilide for *Verticillium* spp.

Relevance criteria:

- Identification of the test species as *Lecanicillium muscarium*
- Subject relevant for toxicological considerations
- Test species relevant to the toxicological assessment
- Route of administration / exposure relevant for assessment
- Endpoint relevant for assessment
- Clinical cases and follow-up studies
- Metabolites or toxins of toxicological concern produced by *Lecanicillium muscarium* Ve6 (19-79)

Reliability criteria:

- Minimum information reported e.g.: test item or related compound, test species relevant
- Clear and comprehensive description of material and methods, including duration, replicates, test conditions

- Definition of endpoints
- Presentation of results
- Guideline compliance

Comment RMS: The RMS questions whether the isolated substances are no important metabolites in the three non-relevant articles according to the applicant. Therefore, RMS requires more explanation from the applicant to explain why these isolated substances are no important metabolites. Furthermore, a comprehensive search of the published literature should be conducted with the aim to find all references regarding the production of toxins or metabolites of toxicological concern. Therefore the applicant should perform a new literature search including the metabolites only or in combination with toxicology (not only the combination of metabolites and the microorganism).

The applicant submitted the following explanation:

-Ishidoh et al. 2014: fungus identified only at genus level: *Lecanicillium* sp. HF627; report is on structure of Verlamelin A and B. No information on its potential effects on human health. Therefore, the report is considered not relevant for the assessment of *L. muscarium* Ve6.

-Shiomi et al., 2010: Verticilide inhibitor of an insect specific RyR (Ryanodin receptor). It binds also to mouse RyR but 10 times weaker than to cockroach RyR. It is produced by *Verticilium sp*. FKI-1033. Produced in culture broth. Taxonomy of the strain is described: due to morphological criteria the strain belongs to the genus *Verticillium Nees*. It seems to be a different species than *L. muscarium* due to different morphology. Therefore, the reference is considered not relevant for the assessment of L. muscarium Ve6.

-Roll et al., 2009: The article presents structural analysis of 4 indolsequiterpenes extracted from a fermentate of *Verticillium lecanii* 6144. One of the substances turned out to be a potent and selective progesterone receptor agonist. Described activities of the substances are of beneficial nature. No further information about possible toxic side effects is provided.

Note RMS: Based on this explanation on different metabolites produced RMS cannot exclude the relevance of these metabolites for *Lecanicillium muscarium* Ve6. Therefore, still the applicant should perform a new literature search including the metabolites only or in combination with toxicology (not only the combination of metabolites and the microorganism).

B.6.1.1.1 Medical data

Information from the original DAR

Open literature

Doekes et al. (2004) described a study in which greenhouse employees were subjected to IgE serology testing at baseline and after 1, 2 and 3 years of follow-up, using a crude high molecular weight of MYCOTAL strain Ve6. Nine to twenty-one percent of the sera tested were positive to *Verticillium lecanii*. For a solid conclusion on the sensitisation-properties of *Verticillium lecanii* Ve6 a more specific IgE detection method is needed in combination with the assessment of work-related symptoms.

Eaton et al. (1986) reported on the skin prick testing programme with *Verticillium lecanii* Ve2 and Ve6 run at Tate and Lyle on 116 employees. A positive reaction (wealing responses) was noted for 4 subjects. At the Glasshouse crops research institute, 31 employees were prick tested, and 4 were scored positive. At both sites, the employees had been exposed to *Verticillium lecanii* for periods for up to 10 years. The positive reactions at both sites were noted for Ve2 or the combination of Ve2 and Ve6 only, no reaction was scored when tested with Ve6 alone.

At Tate and Lyle, 85 employees (including the 4 skin positive ones) were also subjected to a medical programme on several haematological, hepatic and renal parameters, lung function and immunoglobulins. These results revealed no abnormalities, indicative of a non-toxic reaction.

Eaton and Walport (1982).

Healthy subjects who had been exposed to *Verticillium* material (for 6 months to 4 years) were prick tested using extracts of *Cephalosporium acremonium* (Center Laboratories) and *Verticillium albo-atrum* (Hollister-Stier (Dome/Hollister-Stier, Slough, U.K.)).

Respiratory function testing was performed (measuring of Peak Expiratory Flow (PEF), Forced Vital Capacity (FVC) and Forced Expiratory Volume in 1 second (FEV1)) plus a general physical examination. All subjects were negative in the prick tests, and all respiratory and physical examinations were regarded normal.

Baelum et al. (2003)

Symptoms and work conditions of 316 persons working in greenhouse companies using microbiological pesticides in Denmark were obtained by interview at annual examinations during 2 years. Spirometry, bronchial challenge and skin prick test with standard inhalatory allergens were also measured. The use of *Verticillium* was considered not related to any symptoms of sensitization and inflammatory lung

diseases among greenhouse workers, whereas the incidence and prevalence of respiratory symptoms and eye-irritancy for *Bacillus thuringiensis* and *Trichoderma harzianum* was relatively higher.

New data 2016

In the literature search covering the last 10 years and focussing on toxicity or pathogenicity of *L. muscarium* on mammals, one article was identified concerning health effects on workers using microbiological pest control agents.

Baelum et al., (2012) evaluated the health effects of exposure to microbiological control agents used in Danish greenhouses including MPCP containing *Verticillium lecanii*, *Bacillus thuringiensis* subsp. *kurstaki* and *Trichoderma harzianum.* IgE levels were above the detection limit in 53% of the blood samples of exposed workers. The measurement was, however, only qualitative and no differences between exposed and not exposed samples are detectable. Thus, IgE levels and exposure levels do not correlate and no significant changes in respiratory symptoms, lung function or bronchial hyperresponsiveness were detectable. Additionally, prevalence rate ratios among exposed increased only marginally from 1.20 (CI95%: 1.01-1.42) to 1.43 (CI95%: 1.09-1.87) over a 3-year period.

No health related reactions were observed in personnel working with *V. lecanii*-derived products for several years, thus, there is no evidence that *Verticillium lecanii* may cause serious health effects after repeated inhalation exposure in mammals.

Cited references:

KMA 5.1.1/01 – Baelum J, Larsen P, Doekes G, Sigsgaard T. (2012), Health effects of selected microbiological control agents. A 3-year follow-up study; Ann. Agric. Environ. Med., 19, 631-636

Executive summary

INTRODUCTION AND OBJECTIVES: Microbiological control agents (MBCA) are widely used in greenhouses, replacing chemical pesticides. The presented study aims to describe health effects of exposure to three types commonly used: *Bacillus thuringiensis*, *Verticillium lecanii*, and *Trichoderma harzenianum* covering seven different products in greenhouse workers with emphasis on sensitization and respiratory effects.

METHODS: 579 persons aged 17 - 67 years culturing ornamental flowers were included. They were followed for three years with annual examinations including interview about exposure and symptoms, lung function, including bronchial (histamine) challenge test, and blood samples. Direct and indirect exposure for each person and year was estimated by information from respondents and employers. IgE in serum against the 7 products of MCBA was analyzed using an enzyme immunoassay technique.

RESULTS: 65%, 40%, and 78% were exposed to *B. thuringiensis*, *V. lecanii*, and *T. harzenianum*, respectively, while 6, 3 and 3% were handling the products. IgE against *B. thuringiensis* was seen in 53% of the samples and with prevalence rate ratios among exposed increasing from 1.20 (CI95%: 1.01-1.42) to 1.43 (CI95%: 1.09-1.87) over the 3-year period.

As regards *Verticillium lecanii* 36% of samples revealed respective IgEs above the detection limit, but no relation to exposure was seen in any of the runs.

There was no relation between exposure to any MBCA and neither prevalence nor incidence of respiratory symptoms and there was no effect on lung function or bronchial responsiveness.

CONCLUSIONS: Use of *Verticillium lecanii* in greenhouses may give rise to antibody formation while no effect on the occurrence of sensitization, respiratory symptoms or lung function was observed. The persons had a relatively long exposure. Therefore, a healthy worker effect may have influenced the results.

B.6.1.1.2 Medical surveillance on manufacturing plant personnel

Information from the original DAR

See B.6.1.1.1

New data 2016

A new short health surveillance report is submitted by the notifier for renewal of *Lecanicillium muscarium* Ve6 under Regulation (EC) No 1107/2009.

No incidents related to adverse health effects to employees, resulting from exposure to *Lecanicillium muscarium* during production, formulation, and handling of microbial products have been reported (Mikkelsen 2016).

Cited references

KMA 5.1.2/01 – Mikkelsen, H. (2016), Statement concerning hazards to man during the use or handling of *Lecanicillium muscarium* strain Ve6 (19.79) CBS collection no: 10207; Unpublished report without report number.

B.6.1.1.3 Sensitisation/allergenicity observations, if appropriate

Please refer to B.6.1.1.2.

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

Information from the original DAR

- Das et al. (1997) described one case in which *Verticillium* was isolated from a swelling on the arm. This patient had several underlying diseases (one kidney was removed and he received radiotherapy and chemotherapy). The lesion on the left arm responded to antifungal therapy and the swelling disappeared gradually.

- Grandesso et al. (1996) reported on seven patients that suffered from a fungal peritonitis. In one case a *Verticillium* species was identified as the causative agent. All these patients had been treated for bacterial peritonitis and were treated by intraperitoneal antibiotics in the previous two months. Patients were cured by removal of the catheter and by antifungal therapy.

- Shin et al. (2002) described a 50-year-old man who suffered from infectious keratitis caused by a *Verticillium* species, without history of trauma. The patient recovered after antifungal therapy. The author emphasizes that *Verticillium* species are very rare causes of keratitis.

New data 2016

In the literature search covering the last 10 years and focussing on toxicity or pathogenicity of *L. muscarium* on mammals, one article was identified describing the outbreak of *Lecanicillium* species in surgery patients.

Neal et al. (2012) describe the pseudo-outbreak of *Acremonium* and *Lecanicillium* spp. in surgery patients. No adverse effects on human health were reported. *Acremonium* species are associated with a wide spectrum of clinical diseases including localized and disseminated infections in immunoincompetent or - compromised hosts. These fungi are also pathogenic to other fungi and have been used as plant protection agents. *Lecanicillium* species are also pathogenic to both insects and some other fungi and are also used as biocontrol agents. Unlike *Acremonium* this species has never been associated with disease in humans. As both fungi share many morphological features including hyaline colony, cottony texture, arrangement of conidia etc., morphological distinction is challenging. A cluster of *Lecanicillium* and *Acremonium* isolates was recovered from tissues of patients removed during orthopedic surgery at a hospital. Fungus culture had been ordered on a couple of samples.

Firstly, *Acremonium* spp. have been identified in isolates of one patient. In total, from 2008 to 2009, 26 out of 320 samples were positive for fungal growth. Nine samples were originally identified as *Acremonium* species. Four of five case isolates were subjected to DNA sequence-analysis at the CDC. Four out of five isolates were identified as *Lecanicillium lecanii*, Environmental swabs taken from operating rooms and labs were negative for both species. Further characterisation revealed that all *Lecanicillium* samples were identical and hence from the same source. The outbreak was accordingly

considered a pseudo-outbreak (no true infection in patients but only in human samples). The source of contamination could not be clearly identified.

However, recovery of soil fungi that are not traditionally thought to be human pathogens from multiple human samples raises concern about handling and processing of human tissues in hospitals. This cluster also provides the utility of DNA sequence-based fungi identification.

True infection was considered unlikely based on full clinical recovery of all patients without antifungal treatment.

Cited references:

Report KMA 5.1.4/01 – Neal, C. O.S., Deak, E., Chang, L. S., Gilmartin, H., Gade, L., Imanishi, M., Price, C., Brandt, M.E., Chiller, T., Balajee, S. A (2012) Pseudo-Outbreak of *Lecanicillium* and *Acremonium* species in orthopedic surgery patients; Journal of Clinical Microbiology, 50, 4103-4106

Abstract

Acremonium species cause a variety of human infections, while *Lecanicillium* species have not been reported as human pathogens. We describe a pseudo-outbreak involving both organisms, highlighting the role and limitations of molecular methods in the characterization of rare fungal isolates. Repeated isolation of these fungi from patient tissue samples raises concerns about exogenous contamination in the hospital environment.

B.6.1.2 Basic studies

B.6.1.2.1 Sensitisation

Information from the original DAR

Sensitisation studies with micro-organisms will be of limited value, as reactions to foreign proteins (on most micro-organisms) can be anticipated. Therefore all micro-organisms should be regarded as potential sensitisers. However, one study on sensitisation was submitted, and is summarised below.

reference	[redacted] (1982)	exposure :	intradermal and topical induction, topical challenge (occlusive, 24 h)
type of study	Dermal sensitisation study (Magnusson & Kligman)	doses :	5% intradermal induction, 25% topical induction, 25% and 12.5% challenge
year of execution	1982	vehicle :	sterile water
test substance	*Verticillium lecanii spp* Spore content: not indicated	GLP statement :	no

	Strain: not indicated		
route	dermal	guideline :	OECD 406
species	Guinea pig, Dunkin Hartley	acceptability :	Acceptable
group size	20 test, 20 control animals, females only		

Study design

A range-finding study was performed to determine the maximum non-irritating concentration of the test material.

A test group of 20 female guinea pigs was used, of which a region of 6 by 4 cm on the shoulder was shaved. A row of 3 injections were given: 0.1 mL complete Freund's adjuvant, 0.1 mL 5% *Verticillium spp* (in sterile distilled water), 0.05 mL complete Freund's adjuvant and 0.05 mL 5% *Verticillium spp*.

Seven days later a 25% topical induction was applied (using a test substance-saturated filter) on the surface of the same injection site for 48 hours. On day 21 (14 days after topical induction), a challenge concentration of 12.5% or 25% (using a test substance-saturated filter) was applied on the flank for 24 hours.

Twenty control female guinea pigs were used in a similar way, except that distilled water was used instead of the test substance *V. lecanii spp*.

Results

No positive skin reactions were observed in any of the test or control animals in the challenge phase.

Acceptability

The study is considered acceptable.

Conclusion

Under the conditions of this maximisation study, *V. lecanii spp* is not sensitising to the skin of guinea pigs.

New data 2016

No new data is submitted under this point.

As all microorganisms are considered to be potential allergens. the precautionary warning phrase 'Contains *Lecanicillium muscarium* Ve6. Micro-organisms may have the potential to provoke a sensitising reaction' must appear on the label.

B.6.1.2.2 Acute toxicity, pathogenicity and infectiveness

Acute oral toxicity, pathogenicity and infectiveness

Information from the original DAR

Study 1

reference :	[redacted] (1982a)	exposure :	Once by gavage
type of study :	Acute oral toxicity, infectivity and pathogenicity	doses :	3.0×10^8 spores/rat
year of execution :	1982	vehicle :	0.9% Saline
test substance :	*Verticillium lecanii* Ve6: Spore content: 1.5×10^9 spores/g	GLP statement :	no
route :	oral	guideline :	EPA OPPTS 885.3050
species :	Rat, COBS, CD strain	acceptability :	Supplementary
group size :	10 males and 10 females	LD_{50}	> 3.0×10^8 spores/animal.

Study design

The study was designed partly in accordance with EPA OPPTS guideline 885.3050. Ten males and 10 females were treated orally with 3.0×10^8 spores/rat SSP (stabilised spore powder) and sacrificed on day 15 and day 29 (in groups of 5 per sex). The dose preparations were analysed for viable spore content by the sponsor. The body weights were determined pre-dose and weekly afterwards. Food consumption was recorded weekly per cage of 5 animals.

At necropsy, gross necropsy was performed on all animals, macroscopic abnormalities were recorded, and several organs (adrenals, kidneys, liver, lung, spleen, testes, and thymus) were weighed. Microscopic examination was performed on animals sacrificed on day 29 only, and the following organs were examined: adrenals, kidneys, liver, lung and bronchi, mesenteric lymph nodes, spleen, testis and thymus. In addition, the liver, lung, spleen, mesenteric lymph nodes and kidneys were examined for any microbial test substance. For this infectivity testing, tissue samples were taken from 3 males and 3 females on day 15 and day 29.

The study deviated from the guideline on the following aspects: An infectivity study was not performed on day 3 and day 8, brain and blood were not examined. In addition, the study was not per-

formed with inactivated (autoclaved) *Verticillium lecanii* (ASSP) and no saline and or carrier control (SSP formulation without the spores) was used.

In addition, according to the current guideline an oral study should also encompass a concurrent control group of 2 animals per sex housed together with the test groups (a "shelf control" group), and 2 control animals per sex housed separately from the treated group.

Results

The numbers of viable *Verticillium lecanii* spores in the dosing solution were in good agreement with the nominal spore concentrations.

Mortality: none

Symptoms of toxicity: no clinical signs were noted.

Body weight and food consumption: no treatment-related findings.

Macroscopic pathology: no treatment-related findings.

Organ weights: no treatment-related findings were noted.

Microscopic pathology: no treatment-related findings.

Infectivity: no fungus was isolated from any organ.

Acceptability

The method of homogenisation of the organs is not described. The RMS cannot deduce whether a proper method was used to homogenise the tissues, ensuring the release of any fungus from the cells, for microbial examination. Therefore this study is considered unacceptable for the evaluation of pathogenicity and infectiveness. The study is acceptable as supplementary for the evaluation of the acute toxicity.

Conclusion

Under the circumstances of this study, *Verticillium lecanii* Ve6 was not acutely toxic after oral administration to rats. The LD_{50} for orally administered *Verticillium lecanii* Ve6 was set at > 3.0×10^8 spores/animal. No conclusions can be drawn on the infectivity or pathogenicity of *V. lecanii* Ve6.

Study 2

reference	:	[redacted] (1998a)	exposure	:	once by gavage
type of study	:	Acute oral toxicity, infectivity and pathogenicity	doses	:	1.2×10^8 spores/rat (nominal dose; actual dose not known)

year of execution :	1998	vehicle :	Phosphate buffered saline
test substance :	MYCOTAL TGAI (= *V. lecanii* Ve6) Spore content: 9.95×10^9 spores /gram	GLP statement :	The RMS could not assess the validity of the GLP-statement [1)]
route :	oral	guideline :	EPA OPPTS guideline 885.3050
species :	Rat,Crj:CD (SD) IGS	acceptability :	supplementary
group size :	Test: 14 animals/sex/group Control: 2 animals/sex/group		

[1)] The GLP-statement in the English translation of the original Japanese study was not signed.

Study design

The study was designed partly in accordance with EPA OPPTS guideline 885.3050. Fourteen males and 14 females were dosed once orally by gavage with 1.2×10^8 spores/rat, and were necropsied in groups of 3 rats on day 3, day 7, and day 14, and in groups of 5 rats on day 21. The control group of 2 males and 2 females received the vehicle only (phosphate buffered saline (PBS)), and was sacrificed on day 21.

Clinical signs were obtained 1, 3 and 6 hours after dosing and once daily afterwards. Body weights were measured prior to dosing and weekly afterwards.

Faeces samples were collected prior to dosing and on days 1, 3, 7 and 14, and were examined for test microbe content using malt extract agar (MEA) media with streptomycin and incubated at 25˚ C for 5 days. Prior to plating the faeces samples were diluted 1:10 in PBS.

At necropsy 1 mL of blood was drawn from the posterior vena cava, and after macroscopic examination, the following organs were dissected: kidneys, brain, liver, lung, spleen, stomach, duodenum, caecum and mesenteric lymph nodes. The blood and organs were examined for microbial test agent content. Prior to plating (in triplicate) on MAE, the dissected organs were homogenised, and blood samples were treated with anticoagulant (K2-EDTA).

The study deviated from the guideline on the following aspects: The study was not performed with inactivated/autoclaved *Verticillium lecanii*, and no shelf control group was used. According to the current guideline an oral study should also encompass a concurrent control group of 2 animals per sex housed together with the test groups (a “shelf control” group), and 2 control animals per sex housed separately from the treated group.

Results

Mortality: none

Symptoms of toxicology: no abnormalities

Body weight: no treatment-related findings

Pathology: no treatment-related findings

Infectivity: no fungus was detected in the organs and faeces samples (LOQ: < 10^2 CFU/g tissue or mL blood).

Acceptability:

As the dose preparations were not analysed for (viable) spore content, no LD_{50} could be deduced from this study, and only a qualitative conclusion can be obtained.

The method of homogenisation of the organs is not described. The RMS cannot deduce whether a proper method was used to homogenise the tissues, ensuring the release of any fungus from the cells, for microbial examination.

Conclusions:

Under the circumstances of this study, *Verticillium lecanii* Ve6 was not acutely toxic after oral administration to rats at a nominal dose of 1.2×10^8 spores/animal (actual dose not known). No conclusions can be drawn on the infectivity or pathogenicity of *V. lecanii* Ve6.

RMS re-evaluation of the study for the purpose of the renewal

As stated in the original evaluation the homogenisation method of the organs was not described. However, the study by [redacted] (1998a) was carried out in the same lab [redacted] in the same time period as the intravenous study [redacted] 1998b) where the detection of the micro-organism in the test organs after dosing (range 10^2 – 10^5 CFU/g indicated that a proper method of homogenisation is applied. Since it has been shown that test laboratory applied an appropriate homogenisation method in their facility it can be concluded that *Lecanicillium muscarium* Ve6 is not infective or pathogenic after acute oral administration.

New data 2016

No new data is submitted under this point.

Although the previously submitted information had some limitation it was considered to be acceptable to cover current data requirements .

Acute inhalation toxicity, pathogenicity and infectiveness

Information from the original DAR

reference	:	[redacted] (1982a)	exposure	:	4 hours, whole body
type of study	:	Acute toxicity, infectivity and pathogenicity	doses	:	See text
year of execution	:	1982	vehicle	:	-
test substance	:	*Verticillium lecanii* Ve6, Spore content: 1.5×10^9 spores /gram	GLP statement	:	no
route	:	inhalation	guideline	:	EPA OPPTS 885.3150
species	:	Rat, COBS, CD strain	acceptability	:	Supplementary
group size	:	Test: 5/sex/dose Control: 5/sex/dose			

Study design

The study was designed partly in accordance with EPA guideline 885.3150. A group of 5 males and 5 females received a maximum practicable dose that was deduced by the RMS (from an appendix that gives limited information on the aerosol characterisation) to be 180 mg test substance/m^3 (0.18 mg/L). In this inhalation study a fan was placed at the base of the exposure cylinder. The rats were exposed for 4 hours to the test substance through a dust feed mechanism, and the control animals were exposed to the same flow rates without the test substance. These rats were sacrificed on day 15. The body weights were determined pre-dose and weekly afterwards. Food consumption was recorded weekly per cage. At necropsy, gross necropsy was performed and macroscopic abnormalities were noted. Histopathological examination was performed on the following organs: adrenal, kidney, nasal passage, larynx and trachea, liver, lung and bronchi, mesenteric lymph node, spleen, testis and thymus. In addition, the liver, lung, spleen, mesenteric lymph nodes and kidneys were examined for any microbial test substance.

The study deviated from the guideline on the following aspects: Infectivity was not studied on day 3 and day 8. Brain, blood and caecum-content were not examined for microbial test substance. No information was given on whether the animals were fasted prior to dosing or not. The observation period was 14 days instead of at least 21 days. In addition, the study was not performed with inactivated *Verticillium lecanii* Ve6.

Results

Mortality: none

Symptoms of toxicity: poor coating conditions and slight hypoactivity were noted in the test substance exposed males only.

Body weight and food consumption: a reduction in body weight was seen during the first 2 days in the test animals, and decreased food consumption was noted in all test animals over the 14 days of observation. No treatment-related effects were seen in controls.

Macroscopic pathology: no treatment-related findings.

Organ weights: no treatment-related findings.

Microscopic pathology: no treatment-related findings.

Infectivity: no test microbe was isolated from any organ.

Acceptability

In this inhalation study the dose to which the rats were exposed to be poorly defined; the study director does not describe a quantitative dose in the main report, and the dose is described as the MPD (maximal practicable dose). In an appendix that describes the aerosol characterisation some information is provided, but not completely clarified. From this information the RMS deduced a dose of 180 mg/m^3. However, the actual dose to which the animals were exposed to is not clear. As the method of homogenisation was not further described, the RMS cannot deduce whether a proper method was used to homogenise the tissues, ensuring the release of any fungus from the cells, for microbial examination. Therefore, this study is considered unacceptable for the evaluation of infectivity. Due to the other deviations from the guideline, described in the study design, this study is acceptable as supplementary for the evaluation of the acute inhalation toxicity.

Conclusion

Under the circumstances of this study, *Verticillium lecanii* Ve6 was not acutely toxic by inhalation at a dose that is not clearly defined. A conclusion on the infectivity of the test substance cannot be given based on the results of this study.

Re-evaluation of the study for the purpose of the renewal:

The original evaluation on the acceptability of the study is agreed with. It is noted by the RMS that a repeated dose inhalation study is available (see B.6.1.2.5). Although the study was carried out with the formulation a maximum dose level of 1.08×10^9 CFU/m^3 was reached.

New data 2016

No new data is submitted under this point.

Intravenously / intraperitoneal single dose

Intravenously study

Information from the original DAR

Study 1

reference	: [redacted] (1998b)	exposure	: once
type of study	: Acute toxicity, infectivity and pathogenicity	doses	: 1.2×10^7 spores/rat (nominal dose; actual dose not known)
year of execution	: 1998	vehicle	: Phosphate buffered saline
test substance	: MYCOTAL TGAI (= *V. lecanii* Ve6) Spore content: : 9.95×10^9 spores /gram	GLP statement	: The RMS could not assess the validity of the GLP-statement [1)]
route	: i.v.	guideline	: EPA OPPTS guideline 885.3200
species	: Rat,Crj:CD (SD) IGS	acceptability	: yes
group size	: Test: 17 animals/sex/group Control: 2 animals/sex		

[1)] The GLP-statement in the English translation of the original Japanese study was not signed.

Study design

The study was designed partly in accordance with EPA OPPTS guideline 885.3200. The study was not performed with inactivated *Verticillium lecanii* Ve6.

Seventeen males and 17 females were dosed once intravenously on day 0 with 1.2×10^7 spores/rat (spores were formulated in PBS 0.01 M, pH 6.8), and were necropsied (3 animals/sex) immediately after dosing, on day 3, day 7, day 14, and day 21 (5 animals/sex). The control group of 2 males and 2 females received the vehicle only (0.01 M, pH 6.8 PBS), and was sacrificed on day 21.

Clinical signs were obtained 1, 3 and 6 hours after dosing and once daily afterwards. Body weights were measured prior to dosing and weekly afterwards.

At necropsy 1 mL of blood was drawn from the posterior vena cava, and after macroscopic examination, the following organs were dissected: kidneys, brain, liver, spleen, duodenum, caecum and inguinal (or femoral) lymph nodes from all groups except the control group. The blood and organs were examined for microbial test agent content. The dissected organs were homogenised in PBS, using a

homogenizer. Blood samples were treated with anticoagulant (K_2-EDTA). The homogenates and the blood samples were incubated in triplicate on malt extract agar (MEA) at 25˚C for 5 days.

Results

Mortality: none

Symptoms of toxicology: no abnormalities.

Body weight: no treatment-related findings.

Pathology: no treatment-related findings.

Infectivity: test microbe was cultivated immediately after dosing in the following organs: liver and spleen (10^5 CFU/g); kidney (10^3 - 10^4 CFU/g); brain, duodenum, caecum (10^2 - 10^3 CFU/g); inguinal lymph node (10^3 CFU/g); and blood (10^2 - 10^3 CFU/mL).

On day 3 test microbial was detected in one male in the spleen (70 CFU/g).

Afterwards no test microbial was detected.

Acceptability:

As the dose preparations were not analysed for (viable) spore content, no LD_{50} could be deduced from this study, and only a qualitative conclusion could be obtained.

Method of homogenisation of the organs is not described. However, the results obtained immediately after dosing indicate a proper method of homogenisation, and in this case is acceptable to the RMS.

Conclusion:

Under the circumstances of this study, *Verticillium lecanii* Ve6 was not acutely toxic after an i.v. injection at a nominal dose of 1.2×10^7 spores/animal (actual dose not known). It was proven that *Verticillium lecanii* Ve6 did not colonize and was not infective, as no viable fungus was recovered from tissue, except immediately after dosing.

Intraperitoneal studies:

Information from the original DAR

Intraperitoneal studies from open literature

An infectivity study with *Verticillium lecanii* in mice and Guinea pigs performed by Mier et al. (1994) was submitted by the notifier. The article is in Spanish, and therefore not assessable by the RMS. The following information was obtained from the abstract (written in English):

Two *V. lecanii* strains (not further specified) were injected intraperitoneally to mice and guinea pigs, including two control groups: heat-killed fungi and sterile saline. The animals were sacrificed at 8, 30 and 70 days post-injection, and mycological and histopathological studies indicated no infectivity.

A study of Murza (1984) of which only the abstract was submitted by the notifier (the original report probably in Russian), contains very limited information. Murza investigated the oral and i.p. LD_{50} of five fungi, including *V. lecanii* to mice, and concluded that the LD_{50} for oral and i.p. administration was > 10^9 and > 10^7 microbial bodies/mouse, respectively. The selected strains were unable to grow and reproduce in mice.

BASIC STUDIES

Study 1

reference	: [redacted] (1982a)	exposure	: once
type of study	: Acute toxicity, infectivity and pathogenicity	doses	: 0 and 1.2 × 10^8 (SSP1) spores/rat or 0.6 × 10^8 spores/rat, see study design
year of execution	: 1982	vehicle	: Saline
test substance	: *Verticillium lecanii* Ve6 Spore content: 1.5 × 10^9 spores /gram	GLP statement	: The RMS could not assess the validity of the GLP-statement[2)]
route	: intraperitoneal	guideline	: EPA OPPTS 885.3200
species	: Rat, COBS, CD strain	acceptability	: Supplementary
group size	: 15 animals/sex/group at 1.2 × 10^8 spores/rat 5 animals/sex/group at 0.6 × 10^8 spores/rat see study design		

[1)] SSP: stabilised spore powder; [2)] The GLP-statement in the English translation of the original Japanese study was not signed)

Study design

The study was designed in accordance with EPA OPPTS guideline 885.3200. Moreover, an infectivity study was also included in the study.

Fifteen males and fifteen females were treated intraperitoneally (i.p.) with 1.2 × 10^8 spores/rat of stabilised spore powder (SSP). Control groups (of similar size) were treated with saline or autoclaved SSP

(ASSP). The rats were scheduled for sacrifice in groups of 5 at day 8, 15 and 30. The dose preparations were analysed for viable spore content.

Three additional groups of 5 rats/sex/group were treated with viable test substance at 0.6×10^8 spores/rat SSP, saline (vehicle control group), ASSP, or with carrier (SSP-formulation without spores, carrier control group). These additional groups were sacrificed on day 3.

The body weights were determined pre-dose and weekly afterwards. Food consumption was recorded weekly per cage. Blood was drawn for haematological and blood chemistry examination on day 3, 8, 15 and day 30. At necropsy or intercurrent death, gross necropsy was performed and macroscopically abnormalities were noted. Microscopic examination was performed on all animals except for the carrier control group (carrier, day 3 sacrifice) and animals sacrificed on day 30. Histopathological examination was performed on the following organs: adrenal, kidney, liver, lung and bronchi, mesenteric lymph node, spleen, testis and thymus.

In addition, the liver, lung, spleen, mesenteric lymph nodes and kidneys were examined for any microbial test substance. This was performed on the first 3 males and the first 3 females of the animals sacrificed on days 8, 15 and 30. For these infectivity studies tissue sections of 0.5 - 1.0 gram were dissected as sterile as possible, weighed and homogenised in diluted (1:4) Ringers medium. This homogenate was plated in quadruplicate on Malt Extract Agar and incubated at 20 - 24˚C and examined morphologically for *Verticillium* at 4, 7, 10 and 14 days. The residual homogenate was incubated in malt extract broth at 20˚C for 7 days.

The study deviated from the guideline on the following aspects: The examination of haematological and blood chemistry parameters is not a data requirement.

Results:

The numbers of viable *Verticillium* spores in the dosing solutions were in good agreement with the nominal spore concentrations.

Mortality: Three males and 1 female receiving ASSP and 4 females and 1 male receiving SSP (1.2×10^8 spores/animal) died within 2 days of dosing.

Symptoms of toxicity:

Prior to death the following clinical signs were noted for these rats: hypoactivity, hunched posture and pilo-erection. At necropsy distension of the gastro-intestinal tract was noted, and at microscopic exam-

ination peritonitis. In the addition to the clinical signs noted in the decedent i.p. treated rats, hypoactivity, hunched posture and pilo-erection was noted in 30% of the rats that received SSP or ASSP for the first 4 days.

Body weight and food consumption:

The ASSP and SSP exposed groups had a slightly lower body weight on day 2 and or 7 as compared to the saline group. This body weight loss was related to decreased food consumption in the first week Body weight and food consumption in all groups were considered normal thereafter.

Haematology:

On day 3 no changes were noted.

Non-significant increase in white blood cell count and neutrophil count, and a decrease in lymphocytes were noted on day 8 for females treated with SSP. These changes (except the change in lymphocytes) were also noted for males.

On day 15 the following statistically significant changes were noted for both sexes treated with ASSP and SSP: increased white blood cell count, increased neutrophil count and a decrease in lymphocytes. On day 29 no changes were noted.

Blood chemistry:

On day 3 the following changes were noted: reduced total protein for females treated with SSP; decreased albumin values for both sexes treated with ASSP and SSP (females); increased α1-globulin for males treated with ASSP, and decreased α1 globulin for females treated with ASSP and SSP. α2-globulin was increased animals treated with ASSP for both sexes, and SSP (females), β-globulin was increase for ASSP and SSP (males). The albumin/globulin ratio was decreased for ASSP (both sexes) and for SSP (females), and blood urea nitrogen values were decreased for both ASSP and SSP (both sexes). After 7, 14 and 29 days of treatment no treatment-related changes were noted.

Macroscopic pathology:

At macroscopic examination the following was noted: adhesion of a majority of organs (stomach, spleen, peritoneum, diaphragm, liver, GI-tract and/or pancreas) in the abdominal cavity at all intervals of testing (day 3, 8, 15 and 30) for both ASSP and SSP treated animals, as well as the carrier-treated group that was terminated after 2 days of treatment. In some animals yellow/white nodules were seen that were associated with these adhesions.

Organ weights: no treatment-related findings were noted.

Microscopic pathology:

Animals treated with both SSP and ASSP showed chronic peritonitis with abscess formation and adhesions involving many organs in the abdominal cavity developing into foreign body granulomas. This was noted in the intercurrent deaths and in the other animals until day 15. At the level of the lymphoreticular system degenerative cells with vacuolated cytoplasm and pyknotic or lysed nuclei were seen in the mesenteric lymph nodes, spleen, Peyer's patches and in the cortex of the thymus. In the liver areas of necrosis were noted related to treatment with SSP.

Infectivity: no fungus was isolated from the organs.

Acceptability

The method of homogenisation of the organs is not described. The RMS cannot deduce whether a proper method was used to homogenise the tissues, ensuring the release of any fungus from the cells, for microbial examination. Moreover, only a part of the organ was homogenised. Therefore this study is considered unacceptable for the evaluation of pathogenicity and infectiveness. The study is acceptable as supplementary for the evaluation of the acute toxicity.

Conclusion

Effects were observed after i.p injection of *Verticillium lecanii* Ve6 in both its viable and autoclaved form. Four ASSP and 5 SSP i.p. treated animals died with 2 days of dosing, probably due to acute peritonitis. The other effects seen for SSP as well as ASSP-treated rats comprised: changes in haematological parameters at day 8 and 15 (increased white blood cells, increased neutrophils, and decreased lymphocytes), changes in blood chemistry at day 3 (decreased albumin concentration, increased α2-globulin concentration, decreased alb/globulin ratio and a decreased blood urea nitrogen concentration). At macroscopic examination adhesion of several abdominal organs was noted at all intervals of testing. Histopathological examination revealed peritonitis with abscess formation involving many organs in the abdominal cavity.

Under the circumstances of this study signs of peritonitis were seen for the micro-organism in both its viable and inactivated form (ASSP). Therefore, it cannot be concluded that the effects are caused by *Verticillium lecanii* Ve6, but are rather immune-related.

No conclusion can be drawn on the infectivity. The method of homogenisation of the organs is not specified.

Study 2

reference	:	[redacted] (1982b)	exposure doses	:	Once
type of study	:	Acute toxicity, infectivity and pathogenicity		:	0.2 mL of 4.0% w/v (4.5×10^7 spores/mice[1)]), see study design
year of execution	:	1981	vehicle	:	0.9% sterile saline
test substance	:	*Verticillium lecanii* Ve6 Spore content: 5.6×10^9 spores /gram	GLP statement	:	no
route	:	intraperitoneal	guideline	:	Partly in accordance with EPA OPPTS guideline 885.3200
species	:	COBS mice (CD-1)	acceptability	:	supplementary
group size	:	15 females per group			

[1)] nominal dose is calculated by the RMS; actual dose is unknown

Study design

The study was designed partly in accordance with EPA OPPTS guideline 885.3200. Fifteen female mice per group were treated intraperitoneally (i.p.) with 0.2 mL per animal of 4.0% w/v *Verticillium lecanii* Ve6 in the form of stabilised spore powder (SSP), homogenised SSP (not clear to the RMS what is intended to achieve with the homogenisation), autoclaved SSP, carrier SSP (fermented broth without the spores) and saline (control). Clinical signs were obtained once daily for 14 days. Body weights were measured prior to dosing and after 2, 7 and 14 days. Food consumption was recorded for the first 2 days and weekly afterwards. Groups of 5 animals were sacrificed on day 2, 7 and day 14 after dosing.

At necropsy, macroscopical abnormalities were recorded. Histopathological examination was performed on the liver and all abnormalities noted at gross section. In addition, the hepatocyte mitotic index (total number of mitotic figures/10 fields/animal) was examined at days 2, 7 and 14.

The nominal dose was calculated by the RMS: 4.5×10^7 spores/animal.

The study deviated from the guideline on the following aspects: The nominal dose was not indicated in units of micro-organisms, and formulations were not analysed for (viable) spore content. No infectivity study was performed (a Commission Directive 2001/36/EC requirement).

Results

Mortality: none

Symptoms of toxicology: no treatment-related signs were noted

Body weight: on day 2, a slight body weight loss was noted for animals treated with SSP, ASSP and homogenised SSP which related to a decreased food consumption over the first 2 days. Body weight and food consumption was normalised thereafter.

Pathology: macroscopic examination revealed adhesions in the abdominal cavity involving the gastrointestinal tract and/or liver and/or the peritoneum after 7 and 14 days for mice treated with SSP and homogenised SSP, associated with white nodules or areas.

In the liver, pale areas were noted in mice treated with SSP and ASSP after 7 days, and for one SSP mouse after 14 days. In the spleen pale areas and/ or nodules were noted for mice treated with SSP and homogenised SSP after 14 days. After 7 days, an enlarged spleen was noted for SSP mice.

Microscopic examination revealed:

After 2 days for SSP and ASSP mice: acute peritonitis with abscesses/granulomata, focal necrosis in the liver, slight increase in haematopoiesis and sinusoidal chronic inflammatory accumulations.

After 7 days the same microscopic findings were noted as after 2 days affecting more animals per group.

A slight increase in the hepatocyte mitotic index was noted for all treated groups, except the saline control. At day 7 this was noted in the mice treated with SSP, ASSP, clarified SSP and homogenised SSP. This elevation in mitotic index was still present day 14, except in the SSP-treated group.

Acceptability:

As the dose preparations were not analysed for (viable) spore content, no LD_{50} could be deduced from this study, and only a qualitative conclusion could be obtained.

Conclusion:

Under the circumstances of this study, signs of peritonitis were induced by the viable spores, inactivated spores and carrier material (without the spores), after i.p. administration (at a nominal dose of 4.5×10^7 spores/animal (actual dose not known)).

Study 3

reference	: [redacted] (1982c)	exposure	: Once
type of study	: Acute toxicity, infectivity and pathogenicity	doses	: 0.2 mL of 4.0% w/v (6.9×10^6 spores/mice[1)]), see study design
year of execution	: 1982	vehicle	: 0.9% saline
test substance	: *Verticillium lecanii* Ve6 Spore content: 8.6×10^8 spores /gram	GLP statement	: no
route	: intraperitoneal	guideline	: Partly in accordance with EPA OPPTS guideline 885.3200
species	: COBS mice (CD-1)	acceptability	: supplementary
group size	: 5 females per group (7 groups)		

[1)] nominal dose is calculated by the RMS; actual dose is unknown

Study design

The study was designed partly in accordance with EPA OPPTS guideline 885.3200. Five female mice per group were treated with a single intraperitoneally (i.p.) injection of: sterile saline (control), carrier (SSP formulation without spores) substance, stabilised spore powder (SSP) and autoclaved SSP. Clinical signs were observed once daily afterwards for 7 days. Body weights were measured prior to dosing and after 3, and 7 days. Food consumption was recorded weekly. At necropsy after 7 days macroscopical abnormalities were recorded. Histopathological examination was performed on the liver and all abnormalities noted at gross section. In addition, the hepatocyte mitotic index (total number of mitotic figures/10 fields/animal) was examined.

The nominal dose was calculated by the RMS: 6.9×10^6 spores/animal.

The study deviated from the guideline on the following aspects: The nominal dose was not indicated in units of micro-organisms, and formulations were not analysed for (viable) spore content. No infectivity study was performed (Commission Directive 2001/36/EC requirement).

Results

Mortality: none

Symptoms of toxicology: no treatment-related signs were noted

Body weight: a reduced body weight was noted the first 3 days of observation in all treated groups possibly related to reduced food consumption.

Pathology: macroscopic examination revealed adhesions together with pale/white areas and/or nodules in: the abdominal cavity involving the diaphragm, spleen, liver, the gastro-intestinal tract, peritoneum and/or omentum in all treated groups, except the saline control. Microscopic examination revealed: chronic/granulomatous peritonitis with abscess formation, and a slight increase of extramedullary haemopoiesis for all treated groups (including the carrier and inactivated spores). A very slight increase in mitotic index was noted for mice treated with SSP and ASSP.

Acceptability:

As the dose preparations were not analysed for (viable) spore content, no LD_{50} could be deduced from this study, and only a qualitative conclusion could be obtained.

Conclusion:

Under the circumstances of this study, signs of peritonitis were induced by the viable spores, inactivated spores and carrier material (without the spores), after i.p. administration (at a nominal dose of 6.9×10^6 spores/animal (actual dose not known)).

Study 4

reference	:	[redacted] (1982c)	exposure	:	Once
type of study	:	Acute toxicity, infectivity and pathogenicity	doses	:	1 mL of 4.0% w/v (3.4×10^7 spores/rat[1)]), see study design
year of execution	:	1982	vehicle	:	0.9% saline
test substance	:	*Verticillium lecanii* Ve6 Spore content: 8.6×10^8 spores /animal	GLP statement	:	no
route	:	intraperitoneal	guideline	:	Partly in accordance with EPA OPPTS guideline 885.3200
species	:	COBS rat (CD)	acceptability	:	Supplementary
group size	:	5 females per group			

[1)] nominal dose is calculated by the RMS; actual dose is unknown

Study design

The study was designed partly in accordance with EPA guideline 885.3200. Five female rats per group were treated with a single intraperitoneally (i.p.) injection of sterile saline (control) or stabilised spore powder (SSP) in saline. Clinical signs were observed once daily afterwards for 7 days. Body weights

were measured prior to dosing and after 3, and 7 days. Food consumption was recorded weekly. At necropsy after 7 days, macroscopically abnormalities were recorded. Histopathological examination was performed on the liver and all abnormalities noted at gross section. In addition, the hepatocyte mitotic index (total number of mitotic figures/10 fields/animal) was examined.

The nominal dose was calculated by the RMS: 3.4×10^7 spores/animal.

The study deviated from the guideline on the following aspects: The nominal dose was not indicated in units of micro-organisms, and formulations were not analysed for (viable) spore content. No infectivity study was performed (a Commission Directive 2001/36/EC requirement).

Results

Mortality: none

Symptoms of toxicology: no treatment-related signs were noted

Body weight: a reduced body weight was noted the first 3 days of observation the treated group possibly related to reduced food consumption.

Pathology: macroscopic examination revealed adhesions in the abdominal cavity involving several organs associated with white nodules. Microscopic examination revealed: a moderate to marked chronic/granulomatous peritonitis with abscess, and a slight increase of minimal inflammatory sinusoidal/portal/perivascular accumulations. A very slight increase in mitotic index was noted occasionally (in the SSP-treated rats only).

Acceptability:

As the dose preparations were not (concurrently) analysed for (viable) spore content, no LD_{50} could be deduced from this study, and only a qualitative conclusion could be obtained.

Conclusion:

Under the circumstances of this study, *Verticillium lecanii* Ve6 induced signs of peritonitis after i.p. administration at a nominal dose of 3.4×10^7 spores/animal (actual dose not known).

New data 2016

No new data is to be submitted under this point.

Previously submitted information is considered to be acceptable to cover current requirements.

B.6.1.2.3 Genotoxicity testing

Information from the original DAR

According to the data requirements (2001/36/EC), genotoxicity tests are required if the micro-organism produces exotoxins according to point 2.8.

Like all living organisms, *V. lecanii* produces secondary metabolites. Only a limited number of compounds have been described for this and related fungi, however they were not considered to have unacceptable effects on human health and/or the environment. As part of the RAFBCA-project it was documented for the first time that *V. lecanii* Ve6 produces destruxins (dtx) A, B and E; however, the production was dependent on the production process (dtx were only observed in extracts from laboratory scale still liquid production, a type of process not used for commercial scale production), the amounts detected were low, and a large variation between the batches was observed. Moreover, in the RAFBCA project the presence of toxins in the formulation, spores, and food crops was analysed (Skrobek et al., 2005 submitted); see B.7.2.1 for a detailed evaluation of this study. The general conclusion was that no destruxins, considered to be the important toxins produced by *V. lecanii* in laboratory cultures, could be detected in the product MYCOTAL, in the unformulated spores or in tomatoes or cucumbers after application of MYCOTAL (10 times the recommended dose). Furthermore, the mode of action of *V. lecanii* Ve6 is not considered to be based on toxins. It can be concluded that any possible exposure to toxins is expected to be negligible, hence no genotoxicity test are considered necessary.

However, as part of the RAFBCA-project several Ames and Vitotox studies were performed with *V. lecanii* Ve6. These studies are submitted by the notifier and evaluated below.

***In vitro* studies**

A one page poster was provided with information on the Vitotox test with *V. lecanii* (Kouvelis et al., 2004). In a Vitotox test two recombinant *Salmonella typhimurium* test strain are used to determine genotoxicity and cytotoxicity simultaneously. The test is based in the luciferase reporter gene system, Activity is measured by light emission in a luminometer as a function of the genotoxicity of the test compound. A genotoxic compound will initiate reactions that derepress the promoter controlling the luciferase gene. The authors concluded on the poster that crude extract of *V. lecanii*, which offer the advantage of examining mixtures of secondary metabolites produced by BCAs, did not show any mutagenicity or genotoxicity in the Ames or the Vitotox assay. Advantages of the Vitotox test are high throughput (with robotic system throughput of > 800 samples/day), high reproducibility and sensitivity

and a small amount of sample. Moreover, the major benefits of the Vitotox test over the Ames test is that the entire DNA content of the cell functions as target for the toxin to display its effects whereas the Ames test monitors mutations only in genes related to histidine synthesis, and the Vitotox test can simultaneously determine cytotoxicity as well as genotoxicity.

Genotoxicity studies were also performed within the EU- RAFBCA project. Typas et al (2004) studied genotoxicity of *V. lecanii* on a number of different *Salmonella typhimurium* strains and on *E. coli* strains with polar and non-polar extracts of unformulated spores of MYCOTAL and extracts from the product MYCOTAL. No mutagenic effects were found with any of these crude extracts which would contain all possible metabolites. Note that also pure metabolites were tested, including destruxins A, and that no mutagenic effects were found.

No *in vitro* clastogenicity study was performed. Instead an *in vivo* micronucleus test (summarized in section B.6.2.2) was submitted using the oral exposure route. However, as no evidence was provided that the compounds reached the target cells, the negative result of this test is considered questionable. In case the intraperitoneal route was chosen instead of the oral route, systemic availability of relevant compounds in the spore extract would have been of less or no concern. As such the *in vivo* micronucleus test submitted is not considered acceptable.

Additionally, a report on an Ames test was submitted which was summarised and evaluated below.

Type of study: Ames test, plate incorporate method.

reference/	:	Nesslany (2002a and b)
type of study	:	Mutagenicity test on Salmonella typhimurium
year of execution	:	2002
test substance	:	*Verticillium lecanii* Ve6 7.25×10^6 CFU/mL spore suspension was homogenised by sonification and vortexing - the ultrasound sonication was done by Branson B-2200 E1 with a working frequency of 47 kHz +/- 6% for 15 minutes (Info from Mr. F. Nesslany). It was centrifuged (5 min at 4000 rpm) and the supernatant was filtered (0.22 µm filter) and Histidine content was determined at 0 µmol/L. This supernatant was used in the Ames test (see below).
GLP statement	:	yes
guideline	:	According to OECD 471
acceptability	:	acceptable

Indicator cells: Salmonella. typhimurium	End point	Res. +act.	Res. - act.	Activation		Dose range
				Tissue	Inducer	
TA1535	Point mutation	-	-	Rat liver	Arochlor 1254	1, 3, 10, 30, 100 µL/plate
TA1537	Point mutation	-	-			
TA98	Point mutation	-	-			
TA100	Point mutation	-	-			
TA102	Point mutation	-	-			
No precipitation was observed at any dose range tested. The dose range was prepared with water. Second test with S9 was performed using the pre-incubation method						

Acceptability

The study was considered acceptable.

Conclusions

Under the conditions of this test, an extract of the spore solution of *V. lecanii* Ve6 did not induce point mutations in *S. typhimurium*.

New data 2016

In the literature search covering the last 10 years and focussing on toxicity or pathogenicity of *L. muscarium* on mammals, one article was identified concerning mutagenic and cytotoxic effects of fungal biocontrol agents including e.g. *Lecanicillium muscarium.*

Kouvelis et al. (2011) report on the assessment of cytotoxic and mutagenic effects of crude extracts of fungal biocontrol agents, including the active ingredient of MYCOTAL, *L. muscarium* Ve6. In addition, purified metabolites including destruxin A, B, D and E were tested. No mutagenic effects were observed in the Ames and VITOTOX assay on any tested organisms.

Thus, there is no evidence that *Lecanicillium muscarium* Ve6 may cause genotoxic effects in bacteria.

Cited references:

KMA 5.2.3.1/01–Kouvelis, V.N., Wang, C., Skrobek, A., Pappas, K.M., Typas, M.A., Butt, T.M. (2011)

Assessing the cytotoxic and mutagenic effects of secondary metabolites produced by several fungal biological control agents with the Ames assay and the VITOTOX® test; Mutat. Res., 722:1 – 6.

Abstract

The potential genotoxic effects of several pure secondary metabolites produced by fungi used as biological control agents (BCAs) were studied with the Ames *Salmonella*/microsome mutagenicity assay and the Vitotox test, with and without metabolic activation. A complete set of *Salmonella* tester strains was used to avoid false negative results. To detect possible mutagenic and/or cytotoxic effects of fungal secondary metabolites due to synergistic action, crude extracts and fungal cell extracts of the BCAs were also examined. Although the sensitivity of the methods varied depending on the metabolite used, clearly no genotoxicity was observed in all cases. The results of the two assays are discussed in the light of being used in a complementary fashion for a convincing risk-assessment evaluation of fungal BCAs and their secondary metabolites.

Materials and Methods

Besides *Trichoderma harzianum*, several *Beauveria, Gliocladium* and *Metarhhizium spp.*, cosmopolitan *Lecanicillium* species and the MYCOTAL strain *L. muscarium* were tested.

Purified metabolites were beauvericin, oosporein, gliotoxin, swainsonine, cytochalasin C, elsinochrome A and the *Lecanicillium*-derived destruxins A, B, D, E.

Ames assays were based on the original method by Maron and Ames (1983) and performed according to the pre-incubation procedure in presence or absence of S9 mix as a metabolizing system. *Salmonella* tester strains TA98, 100, 1535, 1537, 1538 and LT2hisG46 were employed. Bacteria were incubated at 37°C for 20 min with metabolites or crude extracts. Test samples consisted of 100 µL bacterial suspension (10^8 bacteria/mL), 100 µL test solution, 500 µL S9 or phosphate buffer. Following preincubation 2 mL top agar was added and samples were plated in triplicate on minimal agar and cultured for 2 days at 37°C. Positive and negative controls were employed in parallel.

The Vitotox test -Kit was used to assess genotoxicity and cytotoxicity of metabolites and extracts according to the manufactures instructions. The test kit uses *Salmonella typhimurium* TA104 derived strains and the luciferase reporter gene system. Activity is measured by light emission in a luminometer as a function of the genotoxicity of the test compound. Dilution series of samples and controls were run in parallel.

Results

Putative mutagenic effects were tested at the maximum feasible concentrations. While positive controls clearly showed mutagenic effects, no genotoxicity was seen with either the pure or metabolites or

crude extracts from the nine BCAs examined both in presence or absence of metabolic activation. Only strains TA1535 and 1537 showed a slight background layer of small colonies indicating a possible cytotoxic effect for gliotoxin and destruxin E (dtxE) without S9 mix.

In the Vitotox assay again, none of the extracts or metabolites exhibited any genotoxic effect. However, cytotoxicity was seen at higher concentrations of destruxins B, D, E and the crude extract of *Lecanicillium muscarium* KV01, but not for dtxA or *L. muscarium* KV07.

In both assays no mutagenicity was evident in presence of metabolic activation.

Discussion

Neither pure metabolites nor crude extracts of fungal BCAs proved to be mutagenic in the Ames assay in presence or absence of S9 mix. Crude extracts can be considered equally important as pure secondary metabolites because under natural conditions mixtures of metabolites are secreted by fungi with a potential for synergistic effects.

The Vitotox assay additionally provided information on the cytotoxicity of the test items. As with the Ames test no genotoxicity was seen. Some cytotoxicity was detected with gliotoxin, oosporein and destruxins B, D and E, this was supported by experiments with tester strains TA1535 and 1537 for dtxE and gliotoxin.

This finding is not surprising as these substances are known to exhibit cytotoxic effects on different cell lines.

However, the entomopathogenic fungi tested produce either no or only traces of the above metabolites *in vivo* and even if then quantities are significantly lower, 10^4 – 10^6-fold below those secreted in nutrient rich media as employed in these tests.

Moreover, studies on individual destruxins have shown that there are no effects on human leukaemia cell lines but on insect cells SF9.

Conclusion

In vitro assessment of genotoxic effects by metabolites or crude extracts is a multistep process requiring a screening strategy based on a number of complementary test systems from bacteria to mammals. Both tests employed can provide rapid and reliable results on bacterial mutagenicity of both metabolites and crude extracts.

In both assays no mutagenicity was evident for any strain tested.

B.6.1.2.4 Cell culture study

Information from the original DAR

According to Commission Directive 2001/36/EC, a cell culture study is only applicable for intracellular replicating micro-organisms (such as, viruses, viroids or specific bacteria and protozoa) unless it is demonstrated that the micro-organism does not replicate in warm-blooded animals.

The RMS recognised that the conditions within the body are considered not optimal for germination of the spores, as the fungi germinate on host pests with different conditions from vertebrates (germinations conditions are not completely elucidated). Furthermore, *V. lecanii* was not infective in the studies submitted. A cell culture study for *V. lecanii* Ve6 is therefore considered not relevant.

New data 2016

No new data is to be submitted under this point.

Previously submitted information is considered to be acceptable to cover current requirements.

B.6.1.2.5 Information on short-term toxicity and pathogenicity

Health effects after repeated inhalatory exposure

Information from the original DAR

Although this study was performed with the preparation instead of the active ingredient, the RMS considers the study suitable for the evaluation of the toxicity and infectivity.

reference	:	[redacted] (1991)	exposure	:	6 hours/day, 5 days/week, nose only
type of study	:	28-day inhalation study	doses	:	0, 1, 10 and 100 mg/m^3
year of execution	:	1991	vehicle	:	demi-water
test substance	:	MYCOTAL Spore content: 1.2 × 10^{10} spores /gram	GLP statement	:	yes
route	:	inhalation	guideline	:	OECD-guideline no 412 (testing chemicals)
species	:	Wistar rats (Hsd/Cpb:WU)	acceptability	:	acceptable
group size	:	high dose: 8 per sex mid- and low-dose: 5 per sex	NOAEL		1 mg/m^3 (1.08 × 10^7 spores /m^3)

	/group control: 8 per sex

Study design

The study was designed in accordance with OECD-Guideline 412. Twenty six male and 26 female rats were used in this 28-day inhalation study. The animals were exposed to the test substance in a modified nose-only exposure unit type 8132 (ADG development Ltd, UK). The rats were exposed for 6 hours on weekdays only (thus were exposed for 20 days in this 28-day study).

The test substance was diluted in demi-water, and test atmospheres were generated by using nebulisers. The rats were exposed to MYCOTAL at doses of 0, 1, 10 and 100 mg/m^3. An additional group of 3 rats per sex was treated at 0 and 100 mg/m^3 for the determination of the spore-content in the lungs after a single exposure of 6 hours.

Clinical signs were obtained once daily afterwards for 14 days.

Body weights were measured prior to dosing and weekly afterwards. After 24 days of treatment blood samples were drawn for haematology and clinical chemistry, urinalysis samples were taken on day 23 or 24. The animals were sacrificed after 28 days. At necropsy macroscopical abnormalities were recorded. Histopathological examination was performed on all gross lesions, adrenals, bone marrow, lung with trachea and larynx, nose, lymph nodes and other major organs.

According to the analytical certificate, MYCOTAL contains 1.08×10^{10} spores/gram (92.5% viable), thus 1 mg contains 1.08×10^7 spores. Of the product MYCOTAL 12.3% is spore powder of *V. lecanii* Ve6 (11.3% viable spores). It should be noted that MYCOTAL also contains another proteinaceous component (20%), known to be a (respiratory) sensitiser (see Annex C).

The study deviated from the guideline on the following aspects: The study was performed with the formulated product MYCOTAL and not with the micro-organism only and no infectivity study was performed (Commission Directive 2001/36/EC requirements). The study was not performed with inactivated spores.

Results

Infectivity of the lungs:

After 6 hours of exposure 0.7 - 2.1 $\times 10^5$ colony forming units (CFU viable spores) were detected in the lungs of the high-dose animals.

Mortality: none

Symptoms of toxicology: no treatment-related signs were noted.

Body weight: no treatment-related findings were noted.

Pathology: macroscopic examination revealed grey discolouration and swollen and spongy appearance in the lungs, and enlarged white discoloured mediastinal lymph nodes at 10 and 100 mg/m^3. Microscopically changes were noted in the nasal cavity, the lungs and the mediastinal lymph nodes, most pronounced at 10 and 100 mg/m^3 (indexed as very slight for 1 mg/m^3). The histological changes comprised: rhinitis and /or respiratory epithelial hyperplasia (nasal cavity), slight increase in alveolar perivascular lymphocytes aggregate and diffuse accumulation in alveolar macrophages (lungs) and paracortical lymphoid hyperplasia (medistenial lymph nodes). These changes indicated a local immune reaction (by inhalation) rather than a toxicological effect. However, this cannot be concluded as no control studies were performed that could substantiate this, i.e. no inactivated spores or carrier material was tested. It should be noted that MYCOTAL contains 12.3% V. lecanii Ve6, but also 20% of another proteinaceous compound, known to be a (respiratory) sensitiser (see Annex C).

Acceptability:

Infectivity was not studied in the other organs.

The study was considered acceptable for evaluation of toxicity of the formulation MYCOTAL.

Conclusion:

Under the circumstances of this study, MYCOTAL (containing *V. lecanii* Ve6, but also a large quantity of other proteinaceous material, known to be a (respiratory) sensitiser induced very slight histopathological changes at 1 mg/m^3, which were considered of no toxicological relevance. Therefore, the no adverse effect level (NOAEL) for MYCOTAL in this study was set at 1 mg/m^3.

New data 2016

No new data is to be submitted under this point.

Previously submitted information is considered to be acceptable to cover current requirement s. As the micro-organisms together with other proteinaceous material may have the potential to provoke a sensitising reaction (as considered for all micro-organisms) in the Lina (1991) study the very slight histopathological changes at 1 mg/m3 are considered to be of no concern.

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

Information from the original DAR

After inhalation: Supply fresh air. If required, provide artificial respiration. Keep patient warm. Consult doctor if symptoms persist.

After skin contact: Instantly wash with water and soap and rinse thoroughly. Get medical attentions if irritation develops and persists.

After eye contact: Rinse opened eye for several minutes (~ 15 min.) under running water. If symptoms persist, consult doctor.

After swallowing: In case of persistent symptoms consult doctor.

In case of human infection by *V. lecanii* strain Ve6 treat patient with antifungal therapy like fluconazole (Das et al., 1997; Shin et al., 2002).

New data 2016

No new data is to be submitted under this point.

Previously submitted information is considered to be acceptable to cover current requirements.

B.6.1.3 Toxicity studies on metabolites and relevant impurities

See B.6.1.2.3.

B.6.1.4 Summary and conclusions of Tier I studies

Acute toxicity, LD_{50}/LC_{50} values

Test sub-stance	Species	Route	Level tested (spores/animal)	Toxicity	Infectiveness	Reference/ notifier
Verticillium lecanii Ve6	Rat	oral	3.0×10^8	No treatment-related findings	*	[redacted] (1982a)
MYCOTAL TGAI	Rat	oral	(1.2×10^8) **	No treatment-related findings	Not infective	[redacted] (1998a)
MYCOTAL TGAI	Rat	i.v.	(1.2×10^7) **	No treatment-related findings	Not infective	[redacted] (1998b)
Verticillium lecanii Ve6	Rat	inhalation	1.08×10^9 CFU/m3	No treatment-related findings	*	[redacted] (1982a)
Verticillium lecanii Ve6	Rat	i.p.	0.6×10^8 and 1.2×10^8	Mortality, clinical signs, BW loss, changes in clinical pathology, lesion in the abdominal cavity	*	[redacted] (1982a)
Verticillium lecanii Ve6	Mouse	i.p.	(4.5×10^7) **	BW loss, lesion in the abdominal cavity	Not per-formed	[redacted] (1982b)
Verticillium lecanii Ve6	Mouse	i.p.	(6.9×10^6) **	BW loss, lesion in the abdominal cavity	Not per-formed	[redacted] (1982c)
Verticillium lecanii Ve6	Rat	i.p.	(3.4×10^7) **	BW loss, lesion in the abdominal cavity	Not per-formed	[redacted] (1982c)

* No conclusion can be drawn based on the single study

** nominal dose; actual dose not known

B.6.2 Tier II

B.6.2.1 Specific toxicity, pathogenicity and infectiveness studies

Information from the original DAR

Short term toxicity is not expected, because accumulation of *V. lecanii strain* Ve6 in the body has not been demonstrated. Acute studies did not reveal toxicity and the sub-acute 28-day inhalation study with MYCOTAL demonstrated minimal effects only.

New data 2016

No new data is to be submitted under this point.

Previously submitted information is considered to be acceptable to cover current requirements.

B.6.2.2 *In vivo* studies in somatic cells

Information from the original DAR

Type of study: rat micronucleus test

reference	: [redacted] (2004)		exposure	:	Once daily for 2 successive days
type of study	: Mutagenicity study using micronucleus test in the rat		doses	:	5, 10 and 20 mL/kg
year of execution	: 2004		vehicle	:	CMC 0.5%
test substance	: MYCOTAL *Verticillium lecanii* Ve6: 2.4×10^9 spores/mL		GLP statement	:	yes
route	: oral		guideline	:	OECD-guideline no 474
species	: Rat: Sprague Dawley		acceptability	:	Not acceptable
group size	: 5 per sex per group				

End point	Results
Micronuclei (bone marrow) at sacrifice, 24 hours after last dose	1)
No signs of toxicity were observed	

1) inconclusive

Before administration, the spore suspension was homogenized by sonification using an ultrasound probe and subsequent ultra-turrax treatment. The top dose chosen is the maximum allowable dose by gavage (20 mg/kg).

Acceptability

The breaking of the spores was not confirmed by microscopic examination. However considering the elaborate procedure followed, it is most likely that the method was sufficient to destroy the spores.

The exposure of the test substance was via gavage. No change in the ratio polychromatic/normochromatic erythrocytes was observed. Therefore, insufficient proof is present that the test substance reached the target cells. Overall the study was considered not acceptable.

Conclusions

No conclusions could be drawn on the induction of chromosome aberrations in mammalian cells by the extract of the spores of *Verticillium lecanii* Ve6.

New data 2016

No new data is to be submitted under this point.

B.6.2.3 Genotoxicity – *In vivo* studies in germ cells

Information from the original DAR

No studies required.

New data 2016

No new data is to be submitted under this point.

Previously submitted information is considered to be acceptable to cover current requirements.

B.6.2.4 Summary and conclusions of Tier II studies

L. muscarium Ve6 was not infective in the studies submitted. Accumulation of *V. lecanii strain* Ve6 in the body has not been demonstrated. So no more information on specific toxicity, pathogenicity and infectiveness was necessary. The submitted reports indicate no genotoxic potential of spore- or MY-COTAL extracts, destruxins, or crude extracts.

B.6.3 Summary of mammalian toxicity, pathogenicity and effectiveness and overall evaluation of the active micro-organism

This draft assessment report is submitted for the re-approval of *Lecanicillium muscarium* Ve6. The studies were performed with *Verticillium lecanii* Ve6. *Verticillium lecanii* Ve6 has been reclassified to the new species *Lecanicillium muscarium* (Zare and Gams, 2001). Therefore, the conclusions drawn for *Verticillium lecanii* Ve6 are considered valid for *Lecanicillium muscarium* Ve6.

In the previous DAR the method of organ homogenisation to examine the microbial content in the submitted oral, i.p and/ or inhalation studies was not described. The RMS could not properly evaluate the results obtained, especially when no fungus was detected in the homogenates that were plated. Therefore the infectivity part of the acute studies was considered unacceptable, and the majority of the studies were considered supplementary. However, after re-evaluation of the intravenous study ([redacted] 1998b) and one of the acute oral studies ([redacted] 1998a) it was shown that the laboratory uses an appropriate homogenisation method.

In addition, when a nominal dose was used, that was not confirmed by analysis of (viable) spore content, the studies were evaluated qualitatively for toxicity and no LD_{50} was deduced.

The oral toxicity study was considered acceptable for evaluation of the oral LD_{50} of *V. Lecanii* Ve6 and was considered to be $> 3.0 \times 10^8$ spores/animal. A second study (nominal dose 1.2×10^8 spores/animal) confirmed the non-toxicity seen in the first study and showed that the micro-organism was not infective or pathogenic.

In the acute i.v. toxicity study *V. lecanii* Ve6 was proven non-toxic, and not colonising or infective. Although the method of homogenisation was not described, the RMS accepts the results as immediately after dosing *V. lecanii* Ve6 was detected in the organs.

In the acute inhalation study *V. lecanii* Ve6 was considered not toxic, no LC_{50} can be determined, as the dose used is defined as maximal practical dose, and the measurements of the aerosol concentrations are not elucidated.

Four acute i.p. studies were submitted by the notifier: two rat studies (1.2×10^8 and 3.4×10^7 spores/animal) and 2 mice studies (4.5×10^7 and 6.9×10^6 spores/animal). These studies were acceptable for the evaluation of toxicity, but not for infectivity/pathogenicity.

In the highest dose for rats mortality was seen, preceded with hypoactivity, hunched posture and piloerection. In all studies, macroscopic examination revealed adhesion in the abdominal cavity involving many organs. Microscopic examination revealed granulomateous peritonitis with abscesses. These findings were noted for both the viable spores (SSP) and inactivated autoclaved spores (ASSP). This indicates a non-specific immune response to foreign material, consisting of a suspension of (autoclaved heat-inactivated) spores that was injected into the peritoneum. This is supported by the haematological data, as on day 15 increased white blood cells and increased neutrophils were noted, that ceased by day 30. Overall these (local) effects ceased by day 30, and were considered an acute immune-reaction rather than a toxicity reaction.

Overall on the subject of infectivity of *V. lecanii* Ve6 the conducted studies could not completely elucidate whether the fungus is infective/pathogenic or not. In these studies spores were administered, and no fungus was obtained by culturing the homogenates of the organs. The conditions within the mammalian body are considered not optimal for germination of the spores, as the fungi germinate on host pests with conditions different from vertebrates. Furthermore, *V. lecanii* Ve6 was not infective when administered i.v. Data from open literature indicate that when *V. lecanii* was administered i.p., no infectivity was observed. In addition, the literature submitted on clinical cases of (systemic) infections of *V. lecanii* described immuno-compromised cases (either having received chemo/radio-therapy or being treated with intraperitoneal antibiotics). Taken together the RMS agrees with the notifier that *V. lecanii* Ve6 is not infective and thus not pathogenic.

Genotoxicity

No destruxins, considered to be the important toxins produced by *L. muscarium* in laboratory cultures, could be detected in the product MYCOTAL, in the unformulated spores or in tomatoes or cucumbers after application of MYCOTAL (10 times the recommended dose) (Skrobek et al., 2005 submitted/not published; see B.7.2.1 for a detailed evaluation of this study). Moreover, the mode of action of *V. lecanii* Ve6 is not considered to be based on toxins. Therefore, it can be concluded that any possible exposure to toxins is expected to be negligible, and no genotoxicity test are considered necessary.

Moreover, the submitted reports indicate no genotoxic potential of spore- or MYCOTAL extracts, destruxins, or crude extracts.

Cell culture

As *Lecanicillium muscarium* Ve6 is not an intracellular replicating micro-organism a cell culture study is considered not relevant.

Short toxicity and pathogenicity

A 28-day inhalation study was conducted in which rats were exposed to 0, 1, 10 and 100 mg/m^3 of the formulation MYCOTAL (containing *V. lecanii* Ve6, but also a large quantity of other proteinaceous material, known to be a (respiratory) sensitiser). Based on the very slight changes noted at microscopic examination in the nasal cavity, lungs and the mediastinal lymph nodes the NOAEL was set at 1 mg/m^3.

B.6.4 References relied on

See for the description of the literature search B.6.1.1.

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed* Y/N	Owner **
Annex II Data and Information					
IIM 5.1/01 B.6.1.1	Pfau, W.	2016	LITERATURE REVIEW ON LECANICILLIUM MUSCARIUM VE6 (19-79): TOXICOLOGY Koppert, 2191392-MA-05-01 n/a GLP/GEP: no Published: no	Y New data for active ingredient, not previously submitted nor evaluated	KBS
IIM 5.2/01 B.6.1.1.1	G. Doekes, P. Larsen, T. Sigsgaard, J. Baelum	2004	IgE Sensitization to Bacterial and Fungal Biopesticides in a Cohort of Danish Greenhouse Workers: The BIOGART Study. Institute for Risk Assessment Sciences, University Utrecht, The Netherlands, Department of Occupational and Environmental Medicine, Odense University Hospital, Denmark and Department of Environmental and Occupational Medicine, University of Aarhus, Denmark - American Journal of Industrial Medicine 46: pp. 404-407. 2004. not applicable published report	N	-
IIM 5.2/02 B.6.1.1.1	Eaton, K.K., Hennessy, T.J., Snodin, D.J., McNulty, D.W.	1986	*Verticillium lecanii*, allergological and toxicological studies on work exposed personnel. The Cedar House, Popeswood Road, Binfield, Berks RG12 5AD, U.K. - Annals of Occupational Hygiene, vol 30, No 2, pp 209-217 Not applicable Published	N	-
IIM 5.2/03 B.6.1.1.1	Eaton, K.K., Walport, M.	1982	Investigation on human subjects exposed to *Verticillium lecanii*. Tate & Lyle, group Research and Development, Pilot plant building, Deacon way, Reading, UK. Koppert Beheer B.V V-12 Not GLP Unpublished	N	KBS

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed* Y/N	Owner **
IIM 5.2/04 B.6.1.1.1	Baelum, J., Larsen, P., Sigsgaard, T., Doekes, G.	2003	Sensitization and inflammatory lung disease among greenhouse workers exposed to microbiological pesticides. Department of Occupational and Environmental Medicine, Odense University Hospital, Denmark - ISBN nr.: 87-7904-112-4: Conference on Occupational Health Risks of Producing and Handling Organisms for Biological Control of Pests in Agriculture (eds. A. M. Madsen, J. Eilenberg, A. Enkegaard, N.B. Hendriksen, D.F. Jensen, J.B. Jespersen, J. Larsen), pp. 13. - Published	N	-
IIM 5.2/05 B.6.1.1.1	Baelum, J., Larsen, P., Doekes, G., Sigsgaard, T.	2012	HEALTH EFFECTS OF SELECTED MICROBIOLOGICAL CONTROL AGENTS. A 3-YEAR FOLLOW-UP STUDY -, not applicable Annals of Agricultural and Environmental Medicine, 19(4), 631-636 GLP/GEP: no Published: yes	N New data for active ingredient, not previously submitted nor evaluated	-
IIM 5.2 B.6.1.1.2	Mikkelsen, H.	2016	STATEMENT CONCERNING HAZARDS TO MAN DURING THE USE OR HANDLING OF LECANICILLIUM MUSCARIUM STRAIN VE6 (19.79) CBS COLLECTION NO: 102071 Koppert, not applicable Koppert Biological Systems GLP/GEP: no Published: no	Y New data for active ingredient, not previously submitted nor evaluated	KBS
IIM 2.7.1/01 B.6.1.1.4	Das, D.K., Grover, R.K., Chachra, K.L., Bhatt, N.C., Bibhabati, M.	1997	Fine needle aspiration cytology diagnosis of a fungal lesion of the *Verticillium* species. A case report. Institute of Cytology of Preventive Oncology and the Department of Radiotherapy, Maulana Azad Medical college and Lok Nayak Hospital, and the Department of Microbiology, G.B. Pant Hospital, New Delhi, India - Acta-Cytologica 41(2), pp. 577-582. - Published	N	-

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed* Y/N	Owner **
IIM 2.7.1/02 B.6.1.1.4	S. Grandesso, G. Amici, C. Bocci, A. Mottola	1996	Fungal peritonitis in peritoneal dialysis: a critical review of seven cases. Microbiology Laboratory "S. Maria dei Battuti" Regional Hospital, 31100 Treviso, Italy. - Alpe Adria Microbiology Journal 5, 15-21. - Published report	N	-
IIM 2.7.1/03 B.6.1.1.4	J.Y. Shin, H.M. Kim, J.W. Hong	2002	Keratitis caused by *Verticillium* species. Department of Ophthalmology, Korea University Hospital, Korea University Medical College, Seoul, Korea - Cornea 21(2), pp.240-242. - Published report	N	-
IIM 2.7.1/04 B.6.1.1.4	Neal, C.O.S., Deak, E., Chang, L.S., Gilmartin, H., Gade, L., Imanishi, M., Price, C., Brandt, M.E., Chiller, T., Balajee, S.A.,	2012	PSEUDO-OUTBREAK OF LECANICILLIUM AND ACREMONIUM SPECIES IN ORTHOPEDIC SURGERY PATIENTS -, not applicable Journal of Clinical Microbiology, 50, 4103-4106 GLP/GEP: no Published: yes	N New data for active ingredient, not previ-ously submitted nor evaluated	-
IIM 5.3.1 B.6.1.2.1	[redacted]	1982	Delayed dermal sensitisation study in the Guinea pig. [redacted] Koppert Beheer BV 97/8203 Not GLP unpublished report	N	KBS
IIM 5.3.2/01 B.6.1.2.2.1 IIM 5.3.3 B.6.1.2.2.3 IIM 5.3.4/01 B.6.1.2.2.4	[redacted]	1982a	Acute toxicity and infectivity study in rats (oral, inhalation, intraperitoneal) [redacted] Koppert Beheer BV TAL/6/82 GLP unpublished report	N	KBS
IIM 5.3.2/02 B.6.1.2.2.1	[redacted]	1998a	A single dose toxicity study of Mycotal TGAI administered orally to rats [redacted] Koppert Beheer BV 7L621 GLP unpublished report	N	KBS

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed* Y/N	Owner **
IIM 5.3.4/02 B.6.1.2.2.2	[redacted]	1998b	A single dose toxicity study of MYCOTAL TGAI administered intravenously to rats. [redacted] Koppert Beheer BV 7L622 GLP Unpublished report	N	KBS
IIM 5.3.4/03 B.6.1.2.2.4	[redacted]	1982b	Single intraperitoneal injection study in mice with 14 day observation period. [redacted] Koppert Beheer B.V. TAL/3/82 GLP Unpublished report	N	KBS
IIM 5.3.4/04 B.6.1.2.2.4	[redacted]	1982c	*Verticillium lecanii* single dose intraperitoneal injection studies in mice and rats with 7 days observation periods. [redacted] Koppert B.V. Toxicol report ref. TAL/4/82 and TAL/5/82 GLP Unpublished	N	KBS
IIM 5.3.5/01 B.6.1.2.3.1	Kouvelis, V.N., C.S. Wang, A. Skrobek, S.N. Tavoularis, T.M. Butt and M.A. Typas,	2004	Ames and Vitotox tests: an ideal combination to determine mutagenicity and genotoxicity. Poster presented at the EC-RAFBCA-IBMA-IOBC Workshop: New insights into risk assessment and registration of fungal biocontrol agents in Europe. 30th September 2004, Brussels	N	-
IIM 5.3.5/02 B.6.1.2.3.1	Typas, M.A., Kouvelis,V.N., Tavoularis, S.N., Pantou M., Ghikas D.	2004	RAFBCA - Final report: 01.11.01-31.10.04. Partner 8. University of Athens, Greece. EU- RAFBCA project: Risk assessment of fungal biological control agents. QLK1-2001-01391. Internal report. pp. 55-65. Non-GLP Unpublished report	N	KBS

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed* Y/N	Owner **
IIM 5.3.5/03 B.6.1.2.3	Skrobek, A., Ravensberg, W.J., Ben El Hadj, N., Vey, A., Butt, T.M.	2005	Studies on the production of destruxins by the biological insecticides Mycotal® and Vertalec®. School of Biological Sciences, University of Wales Swansea, Singleton Park, SA2 8PP, UK, Koppert Biological Systems, POB 155, 2650 AD Berkel en Rodenrijs, The Netherlands, Institut National de la Recherche Agronomique (I.N.R.A.), Unité de Recherche de Pathologie Comparée, 30380 St Christol les Alès, France. - - - To be submitted.	N	KBS
IIM 5.3.5/04 B.6.1.2.3.1	Nesslany, F.	2002	Mutagenicity test on *Salmonella typhimurium* His- using B.N. Ames's technique with *Verticillium lecanii* Institut Pasteur de Lille, Genetic Toxicology Laboratory, 1, rue du Professeur Calmette – BP.245, F-59019 Lille Cedex, France Koppert Beheer B.V. IPL-R 020805 GLP Unpublished report	N	KBS
IIM 5.3.5/05 B.6.1.2.3.1	Nesslany, F.	2002b	AMENDMENT NO 1 TO THE REPORT IPL-R 020805 - MUTAGENICITY TEST ON SALMONELLA TYPHIMURIUM HIS-USING B.N. AMES TECHNIQUE WITH VERTICILLIUM LECANII Koppert, IPL-R 020805 Institur Pasteur, France GLP: yes Published: no	N	KBS
IIM 5.3.5/06 B.6.1.2.3.1	Kouvelis, V.N., Wang, C., Skrobek, A., Pappas, K.M., Typas, M.A., Butt, T.M.	2011	ASSESSING THE CYTOTOXIC AND MUTAGENIC EFFECTS OF SECONDARY METABOLITES PRODUCED BY SEVERAL FUNGAL BIOLOGICAL CONTROL AGENTS WITH THE AMES ASSAY AND THE VITOTOX TEST -, not applicable Mutat Res, 722, 1-6 GLP/GEP: no Published: yes	N New data for active ingredient, not previ-ously submitted nor evaluated	-
IIM 5.3.7.2/01 B.6.1.2.5.1	[redacted]	1991	Sub-acute (28-day) inhalation toxicity of Mycotal in rats. [redacted] Koppert Beheer B.V. V91.209 GLP Unpublished report	N	KBS

Annex point / reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner** **
IIM 5.5.2 B.6.2.2	[redacted]	2004	Mutagenicity study using the micronucleus test in the rat with verticillium lecanii, strain Ve6 Koppert, FSR-IPL 040104 [redacted] GLP: yes Published: no	N	KBS

**: Protection for 5 years claimed from date of decision concerning listing in Annex I - the study report has not been submitted any of the Member States in support of an application for authorization, or (though the study report has been submitted) has not been used any of the Member States as the basis for decision on the initial authorization, or to maintain a given authorization, of a plant protection product before the date of submission of the dossier to Rapporteur Member State.*

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