Renewal
Assessment Report

Lecanicillium muscarium Ve6

Volume 3MA – B.8 Fate and behavior in the environment

January 2018

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

Version history

When	What
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B Summary of the data and information

B.8 Fate and behavior in the environment

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 re-classified 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].

Although Zare & Gams reclassified members of *Verticillium* section Prostata IV to *Lecanicillium* and *Simplicillium* gen. nov. already in 2001, some recent publications in the open literature may still use the old designation. As long as no more information on taxonomy is provided, species identity has to be considered carefully.

Lecanicillium muscarium Ve6 is a ubiquitous entomopathogenic fungus, originally isolated in 1979 from the glasshouse whitefly *Trialeurodes vaporariorum.* The formulated product of *L. muscarium* Ve6 is used in greenhouses and tunnels against whitefly and thrips. Since the fungus needs high humidity for effective germination of spores, conditions during application need to be adequate. Therefore it is recommended to apply the product in the evenings in greenhouses and in closed tunnel systems to provide high humidity for at least 12 hours.

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.

Introduction

Due to a recent reclassification (Zare and Gams, 2001, submitted in MA 7.1) the fungus *Verticillium lecanii* Ve6 has been renamed to the new species *Lecanicillium muscarium.* When a study was performed with *Verticillium*

[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

lecanii Ve6, the new name *Lecanicillium muscarium* has been used in DAR Volume 3 B.8. When another strain was studied or the strain was unknown the old name *Verticillium lecanii* is used.

The purpose of this chapter is to identify the potential hazards, to identify potential routes of exposure following uses and emissions — with reference to Volume 3 Annex B.3 ("Data application and further information") — and, finally, to assess whether the extent of survival and distribution of *L. muscarium* in soil, water and air are acceptable according to the Uniform Principles.

Regarding Annex B8 a dossier with various scientific studies and tests has been submitted to support within the 4th review EU program the notification of *L. muscarium.* There are two main groups of data or information used by the RMS.

First, two study reports discussed directly the degradation of spores in soil and water and the laboratory conditions under which these test results were obtained. These confidential study reports will be evaluated and summarised.

Second, various statements, scientific studies and reviews have been submitted that are considered relevant by the RMS for the evaluation and assessment, though in a more indirect way, for instance as only (small) parts were considered relevant and useful for risk assessment. These studies and reviews are generally easily accessible (scientific journals and other publications). On the other hand, other available or submitted studies may have been omitted from the evaluation, because of a less relevant research theme. This has been motivated in the text.

RMS comments on introduction at renewal: origin, properties, survival and residual metabolites of the microorganism to assess its fate and behaviour in the environment

The Microbial Pest Control Agent (MPCA) *Lecanicillium muscarium Ve6* (formerly *Verticillium lecanii* Ve6) is an entomopathogenic fungus that was isolated from the greenhouse whitefly *Trialeurodes vaporariorum*. Based on DNA sequencing data, the species *Verticillium lecanii* was renamed and divided into a number of new taxonomical units, including *Lecanicillium muscarium* (Zare and Gams, 2001).

For the original approval of this MPCA, the notifier has submitted literature that demonstrates the worldwide distribution of *Verticilllium lecanii* (see section B.2.1.2) and the ability of *Verticilllium lecanii* to grow using a broad range of hosts and substrates (e.g., as soil pathogen on other fungi, as a hyperparasite on rust fungi, a as parasite on arthropods, and on plant material). The *species Lecanicillium muscarium* also has a worldwide distribution; e.g., see Zare and Gams (2001), in which strains are described from the UK, Italy, but also a location as remote as New Caledonia. In addition, a strain of *Lecanicillium muscarium* has also been isolated from Continental Antarctica (Fenice and Gooday, 2006). No information is provided on the natural distribution of *Lecanicillium muscarium* Ve6 at strain level.

Lecanicillium muscarium in not known to be a human pathogen or to cause effects on human health (see section B.2.5). *Lecanicillium muscarium* Ve6 has been shown to be able to produce destruxins (see section B.2.7). However, no destruxins were detected in spores, mycelium, colonised rice, filtrates of production-scale cultures, or the end-use product. Destruxins were also not detected in plant material treated with foliar applications of the formulated product Mycotal (at ten times the recommended dose; see reference Butt et al., 2004 in section B.2). Therefore, no metabolites of concern are taken into account for this risk assessment.

Information on the genetic stability of *Lecanicillium muscarium* and the possibility of transfer of genetic material in the environment is provided in section B.2.6.

Cited studies, scientific peer-reviewed open literature and other references:

Reference	Fenice and Gooday (2000) Mycoparasitic actions against fungi and oomycetes by a strain (CCFEE 5003) of the fungus *Lecanicillium muscarium* isolated in Continental Antarctica
Reference number	IIM 7/01
Guideline	non-guideline
Test substance	*Lecanicillium muscarium* CCFEE 5003
Previous evaluation	Submitted by RMS for the purpose of renewal
Source	Annals of Microbiology **56**(1) 1-6
GLP	non-GLP

Abstract A strain (CCFEE 5003) of *Lecanicillium muscarium,* isolated in Continental Antarctica, *showed* mycoparasitism in agar cocultures, at 5 and 25 °C, against *Mucor mucedo, Botrytis cinerea*, *Pythium aphanidermatum* and *Phytophthora palmivora.* Different sequential steps were observed in the process leading to parasitism and resulting in a complete host disruption. Parasitism against fungi was characterised by diffused penetration into the host mycelium; with oomycetes, penetration was less evident and the contact between the two organisms was more intimate. Production of glucanolytic and chitinolytic enzymes appeared related to the mycoparasitic process.

RMS comments on Fenic and Gooday (2006) at renewal

This paper describes a strain of *Lecanicillium muscarium* that was isolated in Continental Antarctica.

B.8.1 Persistence and multiplication

RMS comments persistence and multiplication at renewal

The scientific studies and reviews that were submitted for the original approval of *Verticillium lecanii* provide information on the persistence and multiplication of the group of species formerly known as *Verticillium lecani*, but not specifically for the currently recognised species *Lecanicillium muscarium* or the strain *L. muscarium* Ve6.

When applied by spraying to the leaf surface of *Chrysanthemum*, *Lecanicillium muscarium* Ve2, the CFUs per square millimeter of the leaves decreased from 146 to 0 within a period of 14 days (Gardner et al., 1984). In the expert meeting PRAPeR M3 (22-26 June 2009), a data gap was identified regarding the influence of UV light on the persistence and multiplication of *Lecanicillium muscarium* in the environmental compartments. No data for *Lecanicillium muscarium* were provided by the notifier; in general it is agreed that fungal spores are susceptible to UV light and that exposure will be limited for greenhouse uses.

A new literature search has been performed for the current renewal (Scholze, 2016). The new search did not yield any information on persistence or multiplication of *Lecanicillium muscarium* Ve6 at the species or strain level.

Cited studies, scientific peer-reviewed open literature and other references:

Reference	Groeneveld (2001) The persistency of *Verticillium lecanii*.
Reference number	IIM 7.1/05
Guideline	non-guideline
Test substance	-
Previous evaluation	DAR, 2006
Source	OpdenKamp Adviesgroep; unpublished statement
GLP	non-GLP

General statement by Groeneveld (2001)

With the anticipated spray applications conidia are brought into the air. Under natural conditions conidia are not actively brought into the air: the conidia of *V. lecanii*, located at the ends of phialides (carriers of conidia) in clusters, are surrounded by a slime layer (slime heads), which prevents the conidia from spreading through air. Precipitation onto soil is more likely. Groeneveld (2001) stated that *Lecanicillium muscarium* is a common fungus in nature, of which the spores can be found in the soil, where they naturally survive.

Groeneveld (2001) used several scientific studies/reviews to indicate that soil conditions influence the survival of *L. muscarium* in soil:

Beyer et al. (1997a and b); detailed information is given under DAR Volume 3 B.8.1.1.

Further literature referred to by Groeneveld (2001) does however not give information on the influence of soil conditions on survival.

Grajek (1994): cultivation of *V. lecanii* in solid-state cultures is described. This study does not give information on fate or behaviour of *V. lecanii* in soil. Therefore, this study is not further used.

Luppi Mosca et al. (1976): in this study a survey has been performed on the occurrence of mycoflora in horticultural soil from the botanical garden of the University of Turin. *V. lecanii* was categorized as a characteristic species. Information on fate and behaviour is absent.

Lysek et al. (1986): this study describes the autodehelminthizing capacity of soils in two Mexican localities. The information that *V. lecanii* has some moderate capacity as an ovicidal fungus indicates that this is to some extent a way of survival/propagation in soil. The information is of little value in the evaluation of the fate and behaviour in soil but confirms the general presence of *V. lecanii* in soil.

Meyer (1998): this study evaluates the capacity of *V. lecanii* in the suppression of eggs of the root-knot nematode *Meloidogyne incognita* in tomato. This study is not used for the evaluation of fate and behaviour in soil.

Sermann et al (1996): this very short summary is probably based on detailed information given by Beyer et al. (1997 a and b). Therefore, the information provided in not further used.

Zimmermann (1988): this review gives no details on the fate and behaviour of *V. lecanii* in soil, water or air.

RMS comments on Groeneveld (2001) at renewal

No comments on the statement of Groeneveld. The two cited studies of Beyer (1997a and b) are discussed in section B.8.1.1.

Other studies on persistence and multiplication

Reference	Gardner et al. (1984) Scheduling of *Verticillium lecanii* and Benomyl applications to maintain aphid (*Homoptera*: *Aphidae*) control on Chrysanthemums in greenhouses
Reference number	IIM 7.1/03
Guideline	-
Test substance	*Verticillium lecanii* strain Ve2 (Vertalec)
Previous evaluation	DAR, 2006
Source	J Econ Entomol **77**:514-518
GLP	Non GLP

Study 1

Reference/notifier	:	Gardner et al. (1984)	GLP state-ment	:	no
Type of study	:	degradation study on leaves	Guideline	:	no
Year of execution	:	1983	Acceptability	:	acceptable
Test substance	:	Vertalec (*Verticillium lecanii* strain Ve2)			

Material and methods:

Degradation:

Test concentration:	15 g of formulation per liter (10^8 CFU per g) including Triton X-100 (0.01%) as surfactant. Sprayed until run-off.
Test system:	*Chrysanthemum* foliage
Temperature:	Greenhouse temperatures
Sampling time points:	At days 0, 1, 7, 10, 14, 18 and 21 after application
Method of analysis:	Dilution plating on Sabouraud's dextrose agar + 1% yeast extract. 24 hours of incubation at 25°C

Bioassays:

Test concentration:	15 g of formulation per liter (10^8 CFU per g) including Triton X-100 (0.01%) as surfactant. Sprayed until run-off.
Test system:	Leaf disks (11 mm in diameter) of Chrysanthemum were cut at day 0, 1 to 10, 14, 18 and 21 days post application. Leaf disk were placed individually in petri dishes containing moistened filter paper. 30 *Myzus persicae* aphids were placed on each disk.
Temperature:	Greenhouse temperatures
Sampling time points:	7 days after treatment
Method of analysis:	Assessment of mortality
Description:	A commercial formulation of *V. lecanii* strain Ve2 (Vertalec) was evaluated to determine its persistence on chrysanthemum foliage. Laboratory assays on leaf disk were done in addition to the persistence tests. Mortality of aphids was assessed in a series of aged leaves
Results:	Persistence of *V. lecanii* decreased from 146 CFU/mm^2 at day 0 to 0 CFU/mm^2 after 14 days (see table 8.1.4.a). The half-life of conidia on chrysanthemum foliage at humidity levels ranging daily from ca. 65 to 90% RH was 4.18 ± 0.152 days. Aphid mortality decreased from 90 to 68 from day 0 to day 3. Between days 4 and 14 aphid mortality was between 25 and 3%.

Table 8.1.4.a Persistence of *V. lecanii* strain Ve 2 on leaves and aphid mortality on leaves[a]

Days after application	Viable CFUs/mm^2	Aphid mortality [% of initial aphid number]
0	146	90
1	107	70
2	42	85
3	32	68
4	1	25
5	2	18
6	4	23
7	3	32
10	3	16
14	0	3
18	0	0
21	0	0

a: data are estimations of data presented in a figure.

Remarks by RMS:

Insect mortalities higher than 70% are only obtained when the number of CFUs is higher than approximately 32 CFU/mm^2. The author assumed that the method of plating foliar washes did not accurately assess total CFU activity on the treated foliage as aphid mortality was still present at day 14 although no CFUs were counted on the leaf. RMS believes that the difference is too marginal to make that conclusion. Moreover, shallow dose-mortality responses seem to be typical for fungus-insect interaction (reference in Rombach, 1988) and fungus epizootics are probably triggered by environmental factors such as humidity and temperature rather than by the size of fungus inoculum. The half life of *V. lecanii* strain Ve2 was recalculated using first order nonlinear degradation. The DT_{50} was 1.2 days (r^2 0.97).

The result that conidia applied to plant surfaces by means of an aerial application have a short life span, can be used for the risk assessment.

RMS comments on Gardner et al. (2004) at renewal

This paper describes the persistence of *Lecanicillium muscarium* strain Ve2 on leaf surfaces. The RMS considers it to be supporting evidence that strain Ve6 does not persist for long periods on leaf surfaces upon application.

Lecanicillium muscarium final addendum November 2009: Data gap: 8.2

No information on the influence of UV light on persistence and multiplication of L. muscarium in the environmental compartments was provided.

Reply by notifier:

In general sunlight can have a damaging effect on spores of fungi. There is a strong negative correlation between persistence of spores with cumulative total solar radiation in the field. There are large differences of susceptibility for sunlight between fungal species. *Verticillium lecanii* conidia are susceptible for UV-B radiation (Braga et al., 2002).

In greenhouses, however, a big part of the damaging wavelengths are absorbed and blocked (Burges, 1998). This is the case in greenhouses covered with glass or with plastics, see Annex I (Van der Blom, 1996).

Harmful wavelengths for spores of fungi are 280 - 320 nm (UV-B), 320 – 400 nm (UV-A) are less damaging, but higher in quantity. Greenhouse glass does not transmit any UV-B and only some UV-A (nearly zero at 320 nm, to 90% at 400 nm) (see figure 1). Similarly, plastics do not transmit any UV-B or they absorb >90% (Burges, 1998).

Studies in plastic greenhouses in South of Spain show that about 90% of solar UV-B radiation is reflected or filtered by the plastic covering the greenhouse (Lasa et al., 2007). The same study shows that UV-B measurements within various levels of the canopy of a sweet pepper crop are even lower, whereby the top of the canopy receives a little more UV-B than lower parts of the crop.

This was also reflected by the persistence of a baculovirus that was significant greater on lower parts of the plant. It has also been reported that there are differences between the underside and the upperside of leaves and it can be assumed that the underside receives fewer UV-B radiation than the upperside.

From the above it can be concluded that the underside of leaves receive very little UV-B (only some percentages) in a greenhouse compared to outdoor conditions.

Mycotal is applied for control of whitefly and is particularly targeted towards the nymphal stages which are located only at the underside of leaves. Furthermore, these stages are found in most levels of the plant except in the top of the canopy. Eggs of whitefly are deposited in the top of the plant, but when nymphal stages have developed, the plant has formed new leaves that shade the leaves with nymphal stages. Mycotal contains the conidiospores of *Verticillium lecanii* which are sprayed on the crop. These spores are susceptible to UV-B, but since UV-B penetration in the greenhouse and to the underside of the leaves is minimal, we have seen in many efficacy trials and in commercial applications that the spores are not damaged and effective in infecting whitefly nymphs.

Lasa et al. (2007) also demonstrated that after 5-8 days the persistence of baculoviruses was still over 60%. It is generally known that baculoviruses are very susceptible to UV-B, more than conidiospores of fungi. We can therefore assume that persistence of *Verticillium lecanii* spores is as good or better than that of baculoviruses. With the recommendation of Mycotal to repeat the treatment 2 to 3 times with an interval of 7-10 days persistence is sufficiently adequate to give control of the whitefly population. The effect of UV-B in a greenhouse crop is more or less negligible.

Comments by RMS:

The studies by Braga et al., (2002) and Lasa et al., (2007) mentioned by the notifier were not submitted and not included in the original dossier. In general RMS agrees it is justified to consider fungal spores are susceptible to UV but no specific information was submitted in the dossier. We agree that in greenhouses the influence of UV will be very limited and this will enhance the efficacy of the product. For greenhouse uses the environmental exposure will be limited and the requested information therefore less relevant. However, the list of intended uses includes field application to strawberries.

RMS comments on influence UV light on persistence at renewal

The full references of the studies cited in the notifier's reply are not provided in the final addendum to the DAR (November 2009).

Although no information on the influence of UV light on the persistence of *Lecanicillium muscarium* Ve6 was provided by the applicant, the RMS is of the opinion that no further information is required.

Persistence and stability of metabolites

Like all living organisms, *Lecanicillium muscarium* (*Verticillium lecanii*) produces secondary metabolites. As part of the RAFBCA-project it was documented for the first time that *V. lecanii* 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. In chapter B 7.1.1 a description of the metabolites and their stability in the environment is assessed.

RMS comments on persistence and stability of metabolites at renewal

The persistence and stability of metabolites of *Lecanicillium muscarium* is described in the introduction of this section (B.8).

Reference	Scholze (2016) Literature review on *Lecanicillium muscarium* Ve6 (19.79): Fate and behaviour in the environment.
Reference number	IIM 7.1/11
Guideline	EFSA guidance on the submission of scientific peer-reviewed open literature (EFSA Journal 2011; 9(2): 2092
Test substance	-
Previous evaluation	submitted for purpose of renewal
Source	GAB Consulting GmbH, Heidelberg, Germany (unpublished)
GLP	-

In order to identify scientific peer-reviewed open literature on the active substance *Lecanicillium muscarium* Ve6, which may affect the assessment on human health, animal health and/or the environment, a literature search was conducted (Scholze, 2016). The literature research was conducted on the DIMDI database provided by the German Institute of Medical Documentation and comprised searches in MEDLINE; BIOSIS, CAB and SCISEARCH databases. Search strategy aimed to find all recent (from 2006 onwards) references that are of relevance regarding fate and behaviour of the concerned microorganism or its close relatives. In total, 70 references were evaluated for relevance basing on their title or abstracts. Three references were assessed in detail basing on their full text. One article was identified to be relevant for Section 7, and two articles were excluded after detailed assessment of relevance. The respective article is summarized in Point 8.1.1.

RMS comments Scholze 2016

The literature search is considered acceptable by the RMS. In light of the retrieval of the small number of relevant records after the rapid assessment for relevance (i.e., three), a more elaborate search could have been performed including more general search terms such as 'environment', 'ecology', 'aquatic', and 'terrestrial'.

The literature search is summarized below.

The literature search was conducted on the DIMDI database and comprised searches in MEDLINE; BIOSIS; CAB and SCISEARCH databases (from 2006 onwards).

Keywords used in Scholze 2016: (*Lecanicillium* OR *Verticillium lecanii* OR Mycotal) AND (fate OR behaviour OR proliferation OR leaching OR mobility OR persistence OR colonization OR survival OR dispers?) AND *either* search term 1, 2 or 3.

- search term 1: (water OR ground OR groundwater) NOT (heavy metal)
- search term 2: (air) NOT (heavy metal)
- search term 3: (soil OR rhizosphere OR field) NOT (metal OR biodegrad? OR biotransform? OR solubiliz?)

The objective of the study was to identify scientific peer-reviewed open literature on the active substance *Lecanicillium muscarium* Ve6, which may affect the assessment of the environmental fate and behaviour.

Relevance criteria:

- Property investigated was relevant for data requirements of Regulation (EC) No 1107/2009
- Subject relevant for environmental properties and occurrence of *Lecanicillium muscarium*
- Subject relevant for population dynamics of *Lecanicillium muscarium*
- Test species/system relevant to the environmental fate assessment
- Location of the studies and geo-climatic conditions of the field studies relevant
- Method of application/exposure relevant for environmental fate assessment
- Assessment/evaluation of mechanistic aspects or e.g. synergisms
- Possible to correlate observations to the agronomic use
- Conclusions given in the abstract robust and clear

Reliability criteria:

- Minimum information reported e.g. test item or related compound
- Test species relevant
- Clear and comprehensive description of material and methods, incl. duration, replicates, test conditions
- Definition of endpoints
- Presentation of result
- Guideline compliance

The results of the study selection process are shown in the table below:

Data requirement capture in the search:	n
Total number of summary records retrieved after all searches of peer-reviewed literature	70
Number of summary records excluded from the search after rapid assessment for relevance	67
Total number of full-text documents assessed in detail	3
Number of studies excluded from further consideration after detailed assessment of relevance	2
Number of studies not excluded for relevance after detailed assessment	1

B.8.1.1 Soil

New data 2016

No additional data on the persistence and multiplication in soil is available for *L. muscarium* from peer reviewed open literature.

Instead data from open literature on the related species *L. lecanii* are presented. However, data on this species may not completely transferrable to *L. muscarium*, since fate and behaviour of fungi might differ between species.

In a recent publication by Xie et al. (2015), persistence and viability of *L. lecanii* in Chinese agricultural soil was studied. Soil was artificially inoculated with 10^7 CFU/g soil (high) or 10^5 CFU/g soil (low) respectively. Soil samples were taken in 10-day intervals in the first two month and subsequently one-month intervals for 14 month in total. Each sampling, four 50 g subsamples were collected to a depth of 20 cm and mixed as one composite sample. For each sample, the dry weight of soil and CFU number were determined. For the CFU quantification, 10 g of each soil sample was suspended in water and Tween 80, diluted and spread on Oatmeel Agar (OA).

Within one month, a very rapid decrease of cells was shown for the two inoculum populations. During the following month, the low density remained stable until 10 month after inoculation (~2×10^4 CFU/g), for both inoculation densities 10^7 and 10^5 CFU/g soil respectively. Ten months after inoculation, cell number decreased again. Although it was shown that the fungus could persist for at least 14 months, it was not detectable in the soil 16 months after inoculation.

Findings on the related species *L. lecanii* confirmed the decline of cell number after application of *L. muscarium* Ve6 in previously performed studies.

RMS comments on persistence and multiplication in soil for renewal

Lecanicillium muscarium is a microorganism with a world-wide distribution. No data are provided on natural background levels of *Lecanicillium muscarium* (see also data gap 8.3 of PRAPeR meeting M3). Therefore, predicted environmental density (PED) values of *Lecanicillium muscarium* Ve6 in soil cannot be compared to the natural densities of *Lecanicillium muscarium* in soil.

The study of Hollingsworth (1983) with *Lecanicillium muscarium* Ve6 shows that this strain can persist at least 40 days in soil. No data are provided on the long-term persistence of this strain in soil. Although information on popluation dynamics following application is lacking at the strain level, this information is available for *Lecanicillium* spp. (formerly *Verticillium lecanii*): In the field study of Xie et al. (2015) it is shown that while *Lecani-*

cillium lecanii can persist for more than a year in soil, no *Lecanicillium lecanii* CFUs were detected after 1.5 years.

It is the opinion of the RMS that although information at the strain level would be preferred, the information provided in this section is sufficient to demonstrate that *Lecanicillium muscarium* occurs naturally in soil and that the use of *Lecanicilllium* spp. as MPCA does not lead to persistent populations in densities that are higher than natural densities of *Lecanicillium* spp.

RMS has performed PEDsoil calculations based on the GAP table provided for the renewal (see B.8.1.1.1)

Cited studies, scientific peer-reviewed open literature and other references:

Reference	Hollingsworth (1983) *Verticillium lecanii* degradation and percolation in soil.
Reference number	IIM 7.1.1/04
Guideline	Merkblatt No. 55 Parts I and II of the German Federal Biological Institute for Agriculture and Forestry
Test substance	*Lecanicillium muscarium* Ve6
Previous evaluation	DAR, 2006
Source	Tate & Lyle PLC, Group Research and Development (unpublished)
GLP	-

Study report ACTIVE INGREDIENT *L. muscarium*

Reference/notifier	:	Hollingsworth (1983)	GLP state-ment	:	no
Type of study	:	persistence in soil	Guideline	:	B.B.A. Merkblatt No. 36
Year of execution	:	1983	Acceptability	:	acceptable
Test substance	:	*L. muscarium* (1.9×10^{10} spores of per gram); batch: VE6-57			

Material and methods:

Degradation :

Test concentration:	100 mg active ingredient, equivalent to 1×10^7 spores per 100 g test soil
Test system:	100 g test soil per flask
Temperature:	22 ± 2.0 °C
Sampling time points:	after 0, 4, 8, 16, 24 and 32 days
Method of analysis:	Dilution plating in duplicate on malt extract and Rose Bengal Chloramphenicol agar for 3, 5 and 7 days at 22°C±2°C
Soil Characteristics:	Standard Speyer 2.2 soil: high humus, loamy sand; %OC 2.9; pH 6.1; MWHC 20% Standard Speyer 2.3 soil: medium humus, sandy loam; %OC 1.16; pH 6.0; MWHC 24%.
Test material:	stabilised spore powder (SSP)
Description:	100 mg SSP (1×10^7 spores) was directly incorporated into 100 g test soil (soils 2.2 and 2.3). Soil was brought at 40% MWHC and incubation occurred at 22°C±2°C. A soil sample of 1 g was collected after 0, 4, 8, 16, 24 and 32 days and analysed.

Results:

In the table below the results on persistence in soil of *L. muscarium* are given.

Table 8.1.1.-1 Persistence in soil of *L. muscarium* in soil at ambient temperature

Soil type	Propagules added/g soil	Number of propagules/g soil					
		Day 0	Day 4	Day 8	Day 16	Day 24	Day 32
loamy sand	1×10^7	1.4×10^7	3.0×10^6	2.9×10^6	2.6×10^6	1.9×10^6	2.6×10^6
sandy loam	1×10^7	7.5×10^6	4.0×10^6	3.3×10^6	3.9×10^6	4.0×10^6	4.5×10^6

Following incubation of test soil samples for four days *L. muscarium* counts fell to 30-40% of the initial level. After this rapid decline the recovery was more or less stable between 26-45% after 32 days.

Conclusions: The half life of *L. muscarium* in soil is 4-5 days. About 26-45% of the initial level remained viable after 32 days (corresponding to a decrease of 74 and 55%). These results are used for risk assessment.

RMS comments on study Hollingsworth (1983) for renewal:

The study of Hollingsworth shows a rapid initial decline in CFUs of *Lecanicillium muscarium* strain Ve6 within the first 3 days after application. After this initial period, numbers of CFUs are stable for the remainder of the incubation period (32 days in total) at approximately 2 – 4 x 10^6 CFUs/gram soil for both soils. Note that this study was also submitted under point B.8.2 (Mobility), where a summary of this study regarding percolation of *Lecanicillium muscarium* through soil is given.

Other studies on persistence and multiplication in soil:

Reference	Beyer et al. (1997a) The behaviour of the entomopathogenic fungus *Verticillium lecanii* (Zimm.) Viegas in soil: II. Longevity of *V. lecanii* in soil and mineral wood and the optimization of its survival by addition of promoting organic substances.
Reference number	IIM 7.1/01
Guideline	-
Test substance	*Verticillium lecanii*, strain V24
Previous evaluation	DAR, 2006
Source	Zeitschrift fuer Planzenkrankheiten und Planzenschutz, **104**(1): 65-74 (published)
GLP	-

Study 1

Reference/notifier	:	Beyer et al. (1997a)	GLP statement	:	no
Type of study	:	Viability in soil	Guideline	:	no
Year of execution	:	1995	Acceptability	:	acceptable
Test substance	:	*V. lecanii*, strain V24			

Substance	Soil type	Condition	Dose [CFU/g soil]	T [°C]	OM [%]	pH	MWHC [%]	Duration [d]	Decrease viability [%]
V. lecanii, strain V24	garden soil	aerobic	1.9×10^7	25	15	6.5	7	40	79
V. lecanii, strain V24	garden soil	aerobic	1.9×10^7	25	15	6.5	33	40	99.99
V. lecanii, strain V24	garden soil	aerobic	1.9×10^7	25	15	6.5	60	40	>99.99
V. lecanii, strain V24	garden soil	aerobic	1.9×10^7	25	15	6.5	98	40	>99.99
V. lecanii, strain V24	garden soil	aerobic	1.2×10^7	20	15	6.5	60	40	89
V. lecanii, strain V24	garden soil	aerobic	1.2×10^7	25	15	6.5	60	40	96
V. lecanii, strain V24	garden soil	aerobic	1.2×10^7	30	15	6.5	60	40	>99.99

Material and methods:

Treatments: Effect of soil water content was determined by incubation of spores at 7, 33, 60 and 98% of MWHC (equivalent to 5, 20, 30 and 40% soil moisture). Water losses were compensated weekly. Three 100 mL flasks were used per treatment and time. One mL spore suspension was added to 10 g air-dried soil. The experiment was also performed for the effect of soil temperatures (20, 25 and 30°C) at 60% of MWHC.

Duration: 40 days

Test conditions: Experiment was performed at 25 °C under dark conditions

Endpoints: Decrease of viability

Observations: At 33 and 60% MWHC certain development of sporulating fungal mycelium was observed. At 7% of MWHC no fungal growth occurred. At 98% of MWHC single long hyphae did not sporulate

Results:

A summary of endpoints is given in the table below.

Table B.8.1.1-2 Viability of spores of *Verticillium lecanii* in soils at different water contents (at 25 °C) and at different soil temperatures (at 60% of MWHC). The values reported for day 0 represent the measured CFU concentration in 1 ml spore suspension that was added to 10 gram of soil.

	CFU/g soil		
MWHC	**day 0**	**day 40**	**% decrease**
7%	1.9×10^7	4×10^6 a[1]	79
33%	1.9×10^7	1.8×10^4 b	99.99
60%	1.9×10^7	7×10^3 b	>99.99
98%	1.9×10^7	1×10^4 b	>99.99

Soil temperature	day 0	day 40	% decrease
20 °C	1.2×10^7	1.3×10^6 a	89
25 °C	1.2×10^7	5×10^5 b	96
30 °C	1.2×10^7	316 c	>99.99

1: different letters represent significant differences

Remarks RMS:

Viability of spores is highest under dry conditions. Still a decrease of nearly 80% is observed after 40 days. Under adverse conditions (too wet, too high temperature) viability of spores is negatively affected and spores become unviable after 40 days. The soil condition influences the survival of spores in the soil. Most suitable conditions are 7% of MWHC and 20°C.

The V24 strain was isolated in 1989 from the aphid *Myzus persicae* in Germany (Hetsch et al., 2002; Alavo et al., 2002). The product MYCOTAL is based on the Ve6 strain that was isolated 1979 from the glasshouse whitefly *Trialeurodes vaporariorum*. Although the V24 strain has a different origin than *L. muscarium* and the strains will certainly differ in virulence and other characteristics, RMS believes that data on persistence of strain V24 can be used for risk assessment in addition to the data on persistence in soil available for *L. muscarium*. The influence of soil parameters is probably more important than the differences in persistence between strains.

RMS comments on study Beyer 1997a for renewal:

The study of Beyer et al., 1997a describes the persistence and multiplication of *Verticillium lecanii* V24 in two unsterile soils and in mineral wool. The persistence of strain V24 was higher in soils containing more humus substances. The number of CFUs in two unsterile soils declined during the first 20 days of the incubations, but remained stable during days 20 – 40 days of the incubations. The number of CFUs applied to the soils and present in the soil at the end of the incubations (day 40) were positively correlated.

Growth and multiplication of strain V24 was detectable when organic substances were added to the soils (e.g., soyflour, glycerol, or both). With the addition of organic substances, the number of CFUs increased between 5- and 30-fold within 5 days and remained at this level for at least 40 days.

The duration of the incubations is 40 days; no data are given for longer incubations periods.

Reference	Beyer et al. (1997b) The behaviour of the entomopathogenic fungus *Verticillium lecanii* (Zimm.) Viegas in soil: I. Viability in soil at different ecological conditions.
Reference number	IIM 7.1/02
Guideline	-
Test substance	*Verticillium lecanii*, strain V24
Previous evaluation	DAR, 2006
Source	Zeitschrift fuer Planzenkrankheiten und Planzenschutz, **104**(1): 54-64 (published)
GLP	-

Study 2

Reference/notifier	:	Beyer et al. (1997b)	GLP statement	:	no
Type of study	:	Viability in soil	Guideline	:	no
Year of execution	:	1995	Acceptability	:	acceptable
Test substance	:	*V. lecanii*, strain V24			

Substance	Soil type	Condition	Dose [CFU/g soil]	T [°C]	OM [%]	pH	WHC [%]	Duration [d]	Decrease viability [%]
V. lecanii, strain V24	garden soil	aerobic	1.7×10^7	25	15	6.5	7	40	70.8
V. lecanii, strain V24	garden soil	aerobic	1.7×10^7	25	15	6.5	33	40	99.3
V. lecanii, strain V24	garden soil	aerobic	1.7×10^7	25	15	6.5	60	40	99.8
V. lecanii, strain V24	garden soil	aerobic	1.5×10^7	25	36	6.0	19	40	28.6
V. lecanii, strain V24	garden soil	aerobic	1.5×10^7	25	36	6.0	33	40	83.3
V. lecanii, strain V24	garden soil	aerobic	1.5×10^7	25	36	6.0	60	40	99.0

Material and methods:

Micro-organism	*V. lecanii*, strain V24
Treatments:	Survival of *V. lecanii* was determined in two different garden soil with varying amounts of organic matter (15 and 36% OM). Effects were determined by incubation of spores at 33% and 60% of MWHC. Water losses were compensated weekly. Three 100 mL flasks were used per treatment and time. One mL spore suspension was added to 10 g air-dried soil. The experiment was also performed for the effect of soil temperatures (20, 25 and 30 °C) at a constant soil moisture rate of 30% (MWHC 60%)
Method of analysis:	Shaking of soil with sterile tap water. The solution was diluted and 0.1 ml aliquots were spread on selective agar plates. The V.lecanii colonies were counted after 7 and 10 days incubation at 25 ºC in the dark.
Duration:	40 days
Test conditions:	Experiment was performed at 20 °C under dark conditions
Endpoints:	Decrease of viability

Results:

A summary of endpoints is given in the table below

Table B.8.1.1-3 Viability of spores of *V. lecanii* in two garden soil with different amounts of organic material and different WHC

	CFU/g soil				
	Soil 15% OM				
MWHC	day 0	day 5	day 20	day 40	% decrease at day 40
7%	1.2×10^7	7×10^6	4.0×10^6	3.5×10^6	70.8
33%	11×10^6	3×10^6	0.2×10^6	0.08×10^6	99.3
60%	11×10^6	0.6×10^6	0.02×10^6	0.02×10^6	99.8
Soil 36% OM					
MWHC					
19%	5.6×10^6	4×10^6	4×10^6	4×10^6	28.6
33%	6×10^6	4×10^6	2×10^6	1×10^6	83.3
60%	6×10^6	2×10^6	0.1×10^6	$0. \times 10^6$	99.0

Viability of spores is highest in the soils with the higher OM content. This effect was observed at the lower soil moisture contents. At 60% MWHC persistence is too low to observe any positive effects of other soil factors.

Additional experiments were performed with the addition of glycerol and soy flour. These additions resulted in an initial strong growth of *V. lecanii* during the first 5 days of the experiment. Numbers of CFU remained stable for the further 35 days of the experiment.

A last variant was the growth of the fungus on moist mineral wool with and without the supplements of soy flour. Without supplements growth of the fungus was not detected. With soy flour the fungus showed an intense mycelium growth and sporulation on the surface of the mineral wool.

B.8.1.1/02 Incubation of *V. lecanii* in mineral wool (Lecanicillium muscarium final addendum November 2009: open point 8.3)

Cylinders of 3.8 cm in diameter and 2.7 cm height were cut from mineral wool and placed in glass beakers of the sam diameter in such a manner that the surface protrudes a few millimetre above the beaker. Mineral wool was inoculated with 15 ml of a spore suspension (2.5×10^7 spores/ml). Mineral wool was carefully pressed to the base of the beaker and the vessel was covered with aluminium foil. Water losses were compensated weekly.

Table 1 Some physical parameters of the soils used
Tab. 1 Einige physikalische Parameter der verwendeten Böden

Parameter	Soil 1	Soil 2
Dry soil density (g/l)	502	810
Humus content (% of the dry matter KANDELER 1993)	36	15
Maximum water capacity or water-holding capacity (WHC, ÖHLINGER 1993) (g water/100 g dry soil):	133	72
pH	6.0	6.5

Particles > 2 mm were removed by sieve

Table 2. Water potentials and percentages of the water-holding capacity at different soil water contents of the soils used
Tab. 2. Wasserpotentiale und prozentualer Anteil an der maximalen Wasserkapazität bei verschiedenen Wassergehalten der zwei verwendeten Böden

Soil water content (g water per 100 g wet soil)	Soil water potential	Percentage of water-holding capacity (WHC)
Soil 1		
20 %	–1.0 MPa	19 %
30 %	–1.5 MPa	33 %
44 %	–0.8 kPa	60 %
Soil 2		
5 %	< –1.0 MPa	7 %
20 %	–850 kPa	33 %
30 %	–0.5 kPa	60 %

Samples were taken at test begin and after 5, 10, 14, 20, 28 and 40 days by removing a 1 cm thick upper and lower layer. The material was disrupted and shaken with sterile tapwater for determination of cfu.

Results

V.lecanii decreased with time from mineral wool in the same manner as unsterile soil. No differences were observed between upper and lower layer. In one variant where soyflower was added the fungus showed an intense mycelium growth and sporulation on the surface.

Table 4 Promotion of *Verticillium lecanii* in soil (cfu/g soil in millions) by the addition of soyflour at different concentrations (soil dry matter basis). Inoculation with blastospores (1.6×10^6 spores/g soil); standard deviation < 0.5 %

Tab 4 Förderung von *V. lecanii* im Boden (cfu/g Boden in Mill.) durch Zugabe von Sojamehl in verschiedenen Konzentrationen (Trockenmassebasis) Inokulation mit Blastosporen (1.6×10^6 Sporen/g Boden); Standardabweichung < 0.5 %

Incubation (days)	*V. lecanii* × 10^6 cfu/g soil				
	Without addition	Addition of soyflour			
		0.1 %	0.25 %	0.5 %	1 %
0	0.6	0.6	0.6	0.6	0.6
5	0.4	3	8	18	29
10	0.4	3	11	20	56
14	0.3	6	13	26	54
20	0.3	5	11	29	41
28	0.2	5	13	23	28
40	0.2	4	12	18	19

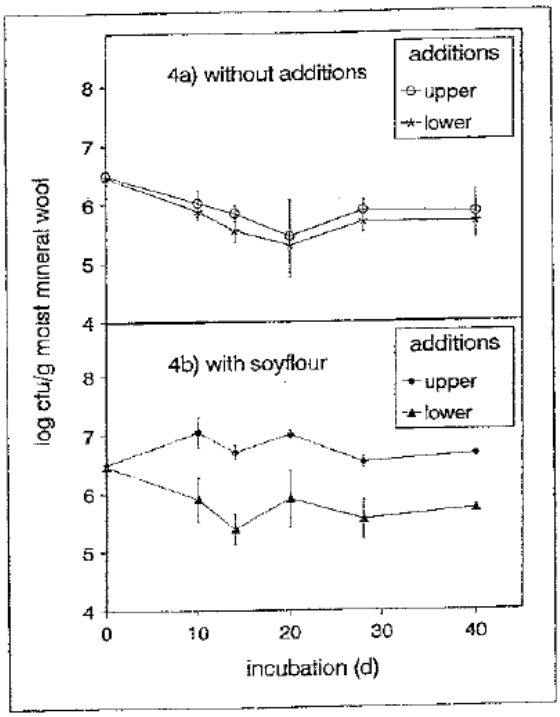


Fig 4 Behaviour of *Verticillium lecanii* in mineral wool (cfu/g wet weight) without (4a) and with addition of soyflour (4b). Cfu-values in the surface layer of the variant with soyflour were significantly higher than in the lower layer and in both layers of the control

Abb. 4 Verhalten von *V. lecanii* in Mineralwolle (cfu/g Feuchtgewicht) ohne Zusätze (4a) und mit Zusatz von Sojamehl (4b). Die cfu-Werte in der Oberschicht der Variante mit Sojamehl waren signifikant höher als in der Unterschicht und als in beiden Schichten der Kontrolle.

Conclusion

The survival of *V.lecanii* in common artificial plant substrate was comparable to that in soil.

Remarks RMS:

The decrease of numbers of CFU without addition of supplements is comparable the results of the preceding study of Beyer et al. (1997a). With supplements the CFU numbers are stable for the duration of the experiment. These experiments, however, did not last long enough. It is assumed by the RMS that CFU numbers will decline in due time and that the effect of supplements is temporarily. This study confirms that a high content of organic matter favours the survival of *V. lecanii* in soil. This result is used for risk assessment.

RMS comments on study Beyer 1997b for renewal:

The change in number of CFUs of *Verticillium lecanii* strain V24 during 40-day incubations were determined for several incubation conditions (soil temperature, soil moisture, pH, soil sterilisation). In general, numbers of CFUs declined during the incubation period, with the most rapid decline at the start of the incubation. For all incubation conditions, strain V24 still detected at the end of the 40-day incubation period.

Multiplication of strain V24 was shown for a sterilized soil with a high content of organic matter. This is attributed by the authors to the lack of competition and antagonism of bacteria and protozoa due to sterilization. However, as the soil were sterilised by autoclaving and autoclaving is known to lead to a higher availability of easily degradable organic matter, this can also have attributed to the growth of strain V24.

Reference	Hall (1981) Laboratory studies on the effects of fungicides, acaricides and insecticides on the entomopathogenic fungus, Verticillium lecanii.
Reference number	IIM 7.1.1/02
Guideline	-
Test substance	*Verticillium lecanii*
Previous evaluation	DAR, 2006
Source	Entomologia Experimentalis et Applicata **29**: 39-48 (published)
GLP	-

Study 3

Tests on agar were performed by Hall (1981) with several fungicides, insecticides and acaricides in combination with Vertalec (a.s. *V. lecanii* strain Ve2). The information provided in this publication concerns the compatibility with chemicals. The virulence of spores sprayed onto plants was indicated by aphid mortality. In the control 100% mortality was obtained 7 days after spraying and 16-35% 5 days after spraying. In this study spores were not recovered from the soil. This study however shows that persistence on plants declines as a decrease of aphid mortality was observed. The results are not used for risk assessment.

Reference	Rombach & Gillespie (1988) Entomogenous Hyphomycetes for insect and mite control on greenhouse crops
Title	IIM 7.1.1/06
Guideline	-
Test substance	-
Previous evaluation	DAR, 2006
Source	Biocontrol News & Information **9**(1): 7-18 (published)
GLP	-

Study 4

The publication of Rombach and Gillespie (1988) did not give relevant data on persistence and is not further used.

RMS comments on Rombach & Gillespie (1988) at renewal

No information on the persistence and multiplication of *Verticillium muscarium* in soil is given in this review paper by Rombach and Gillespie (1988). Note that this paper has also been submitted under point B.8.2 (Mobility).

Reference	Xie et al. (2015) Persistence and viability of *Lecanicillium lecanii* in Chinese agricultural soil
Reference number	IIM 7.1.1/07
Guideline	-

Test substance	*Lecanicillium lecanii*
Previous evaluation	Submitted for the purpose of renewal
Source	PloS ONE, 10, e0138337 (published)
GLP	-

Abstract: The entomopathogenic fungus *L. lecanii* has been developed as biopesticides and used widely for biological control of several insects in agricultural practice. Due to the lack of isolation/count methods for *L. lecanii* in soil, the persistence of this fungus in soil appears to have attracted no attention. A selective medium and count method for *L. lecanii* in soil based on cetyl trimethyl ammonium bromide (CTAB) was developed, and then the persistence and viability of this fungus in soil were investigated under field conditions between 2012 and 2014. The results showed that the rate of recovery for *L. lecanii* in soil on the selective CTAB medium was satisfactory. The minimum CFUs for *L. lecanii* on the selective medium (0.5 g/L CTAB) was about 10^2 conidia/g soil. The *L. lecanii* density in soil declined quickly in the first month after inoculation with fungal conidia, kept stable for 6 to 10 months, and then decreased gradually until undetectable. *L. lecanii* could persist for at least 14 months in the agricultural soil of northern China. The colony growth, conidia yield and germination rate on plates, as well as the median lethal concentration or times (LC_{50} or LT_{50}) to aphids, mycelium growth in aphids and sporulation on aphids of *L. lecanii* did not change significantly during the persistence in soil. In general, the count method developed here was a very useful tool for monitoring the dynamics of natural or introduced *L. lecanii* populations in soil, and the data on the persistence of *L. lecanii* in soil reported here were helpful for biological control and environmental risk assessment.

RMS comments on Report KMA 7.1.1/01; Xie et al., 2015:

A selective medium for entomopathogenic fungi was developed on which colonies of *L. lecanii* can be easily identified from those of *B. bassiana*, *M. anisopliae* and *P. lilacinus*. The persistence of *L. lecanii* in soil was studied in 2 field trials. No *L. lecanii* CFUs were detected in the soils prior to application. Upon application, the number of *L. lecanii* CFUs in soil declined during a two-month period, then remained stable at densities of approximately 2 - 4 x 10^4 CFUs per gram soil for several months, irrespective of the application density. No CFUs of *L. lecanii* were detected in the two soils after 15 and 17.5 months after application.

This report is considered reliable and relevant by the RMS.

B.8.1.1.1 Predicted environmental concentrations in soil

Lecanicillium muscarium final addendum November 2009: Open point: 8.8 **: B.10.2.2 calculation of PECsoil**

Considering the applications rates in cucumber, tomato, sweet pepper and ornamentals of 0.322 kg a.s./ha (equivalent to 2 x 10^{13} CFU/ha) and the application rates in strawberry and ornamentals of 0.161 kg a.s./ha (equivalent to 1 x 10^{13} CFU/ha) the initial PEC can be calculated. Assuming a distribution of the a.s. over the upper 5 cm of the soil layer and a soil density of 1500 kg/m^3, no degradation and no interception, PEC values as reported in the following table are calculated.

Crop	**Kg a.s./ha**	**CFU/ha**	**Max. number of applications**	**PEC_{ini} after 1 appl.**	**$PEC_{max,initial}$ mg a.s./kg**	**$PEC_{max,initial}$**

				CFU/kg soil[1]	**soil[1]**	**CFU/kg soil[1]**
ornamentals	0.322	2×10^{13}	12	2.7×10^{7}	5.15	3.2×10^{8}
cucumber, tomato, sweet pepper	0.322	2×10^{13}	12	2.7×10^{7}	5.15	3.2×10^{8}
strawberry[2]	0.161	1×10^{13}	12	1.3×10^{7}	2.58	1.6×10^{8}

[1] assuming a distribution over the top 5 cm of the soil and 1500 kg soil/m^3

[2] the PEC determined for strawberry is the most relevant as this is a field crop. The other crops are greenhouse crops and are frequently grown on artificial medium.

These PECsoil values may be compared to background concentrations if available.

PEC values are calculated as follows:

A dose rate of $2x10^{13}$ cfu/ha is equal to a dose of $2x10^{13}$ cfu/10000 m^2 which is again equal to a dose rate of $2x10^{9}$ cfu/m^2.

For the calculation of the amount of cfu/kg soil we assume a soil depth of 5 cm (0.05 m). 1 hectare of soil, considering a depth of 5 cm and a soil bulk density of 1500 kg/m^3 has a mass of 750000 kg. 1 m^2 of soil considering 5 cm depth has a mass of 75 kg (75 kg/m^2). This means a dose rate of $2x10^{9}$ cfu/m^2 is equal to $2x10^{9}$ cfu/75 kg is equal to $2.67x10^{7}$ cfu/kg soil.

12 applications with no interception and no degradation between applications the total amount in soil (PIEC) is 12 x $2.67x10^{97}$ = $3.2x10^{8}$ cfu/kg soil.

RMS comments on PEDsoil calculations for renewal

RMS has performed PEDsoil calculations based on the GAP table provided for the renewal; the results are given in Table 8.1.1-4. For the calculations 0% crop interception and no degradation or growth of the MPCA between applications was assumed. As worst case total yearly applications are dosed at once. For greenhouse uses the PEDsoil are not required for the assessment (for completeness, also the greenhouse uses are included in Table 8.1.1-4).

Table 8.1.1-4 PEDsoil calculations for *Lecanicillium muscarium* Ve6 (5 cm soil depth)

Crop	**F/G***	**g a.s./ha per application**	**CFU/ha per application**	**Max. number of applications**	**PED_{ini} after 1 appl. CFU/kg soil[1]**	**$PED_{max,initial}$ mg a.s./kg soil****	**$PED_{max,initial}$ CFU/kg soil[1]**
fruiting vegetables of *Cucurbitaceae*	G	96	2×10^{13}	12 per use; 36 per season	2.67×10^{7}	1.54 per use; 4.61 per season	3.2×10^{8} per use; 9.6×10^{8} per season
fruiting vegetables of *Solanaceae*	G	96	2×10^{13}	12	2.67×10^{7}	1.54	3.2×10^{8}
strawberry	G	48	1×10^{13}	12 per use; 24 per season	1.33×10^{7}	0.768 per use; 1.54 per season	1.60×10^{8} per use; 3.20×10^{8} per season
strawberry	F	48	1×10^{13}	12 per use; 24 per season	1.33×10^{7}	0.768 per use; 1.54 per season	1.60×10^{8} per use; 3.20×10^{8}

							per season
floriculture crops, except cut roses	G	96	2×10^{13}	4 per use; 24 per season	2.67×10^{7}	0.512 per use; 3.07 per season	1.07×10^{8} per use; 6.4×10^{8} per season
cut roses	G	144	3×10^{13}	24	4.00×10^{7}	4.61	9.60×10^{8}
tree nursery crops	G	96	2×10^{13}	24	2.67×10^{7}	3.07	6.4×10^{8}

* F = field application; G = greenhouse application

** assuming a distribution over the top 5 cm of the soil and 1500 kg soil/m^3

B.8.1.2 Water

The applicant made the statement (Koppert Beheer B.V., 2004) that *V. lecanii* does not belong to the resident flora of water derived from groundwater, because the species does not persist long enough. The fungus has a half-life of several days only. This statement referred to the publication of Göttlich et al. (2002) who monitored that presence of *V. lecanii* in public drinking water. The fungal flora in groundwater-derived public drinking water in Germany was determined. *V. lecanii* was exclusively found at locations in the drinking water system where newly buried pipes had been put into service. *V. lecanii* probably infected the water in newly buried pipes by soil or infected arthropods before the pipes were closed. The applicant concluded that the generally occurring soil fungus *V. lecanii* does not belong to the resident fungal flora of groundwater-derived public drinking water.

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.

From peer-reviewed open literature no additional references were identified to be relevant for persistence of *L. muscarium* Ve6 in water. Please refer to the literature search submitted in Point MA 7.1. Therefore, there is no indication for *L. muscarium* population in water. *L. muscarium* is no natural habitant of water. Furthermore, the fungus has a half-life of several days only (please see previously submitted information). This is confirmed by the toxicity study on fish, where CFU count was documented during the 96 h test (please refer to the acute toxicity study to rainbow trout, previously submitted in Doc M-MA, Section 8, Point MA 8.2.1). It was shown that the number of cells was reduced at 2-10 fold already within 96 hours.

RMS comments persistence and multiplication in water for renewal

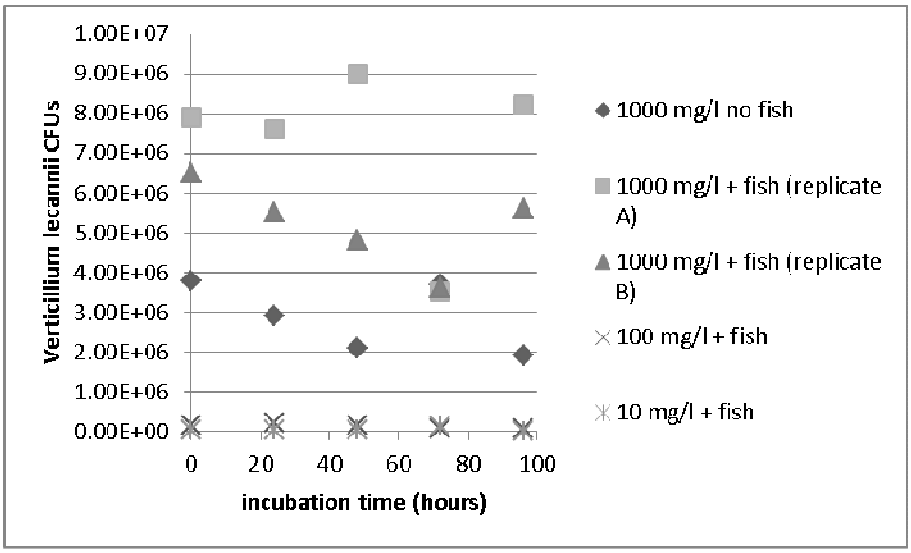
The notifier states that *Lecanicillium muscarium* is no natural habitant of water. Indeed, neither the original submission nor the publications that were retrieved with the literature search that was performed for the renewal included data on the natural occurrence of *Lecanicillium muscarium* in aquatic systems. However, absence of proof is no proof of absence.

The study on the behaviour of *Verticillium lecanii* spores in water (Van der Pas, 2000) shows that 95% of *Lecanicillium muscarium* spores remain viable in stirred sterile demi-water during a 7-day period and that mycelial mats of *Lecanicillium muscarium* can form in unstirred incubations.

The notifier makes reference to the acute toxicity study to rainbow trout in which the CFUs in water was monitored during 4 days after application of *Ventricillium lecanii* ('Ve6-58'; previously submitted in Doc M-MA, Section 8, Point MA 8.2.1). In contrast to the statement of the notifier, no trend in the CFU numbers is observed

over the incubation time, except for the control incubation without fish (see Figure 8.1.2-1). As no replicates were used for this experiment, no conclusions can be drawn from this difference between the incubations with and without fish (such as for example reduced availability of organic compounds in the incubations without fish or a higher rate of attachment of the fungus to surfaces). However, from the CFU data of the toxicity study it can be concluded that CFU numbers of *Ventricillium lecanii* in an aquatic environment remain stable for the period of 4 days.

Figure 8.1.2-1 CFU numbers of *Ventricillium lecanii* ('Ve6-58') during the acute toxicity to rainbow trout



To quantify the persistence and multiplication of *Lecanicillium muscarium* Ve6 in aquatic systems, replicated tests in several water/sediment systems are necessary, in which the population dynamics are monitored over sufficiently long periods.

No data have been provided by the applicant on the persistence and multiplication of *Lecanicillium muscarium* Ve6 in sediment.

As *Lecanicillium muscarium* has a cosmopolitan distribution, aquatic systems are exposed to this microorganism under natural conditions. Even if *Lecanicillium muscarium* is capable of growth in natural aquatic systems, it is the opinion of the RMS that the potential population size will depend more on the conditions in the aquatic system (such as availability of organic matter) than on the additional exposure of aquatic systems due to the use of *Lecanicillium muscarium* as MPCA.

RMS has performed PEDsw calculations based on the GAP table provided for the renewal (see B.8.1.2.1)

Cited studies, scientific peer-reviewed open literature and other references:

Reference	Koppert Beheer B.V. (2004) Persistence and multiplication of *Verticillium lecanii* in water.
Reference number	IIM 7.1.2/02
Guideline	-
Test substance	-
Previous evaluation	DAR, 2006

Source	Koppert Beheer B.V., Department R&D Microbials and Regulatory affairs (unpublished statement)
GLP	-

Göttlich et al (2002) determined the fungal flora in groundwater-derived public drinking water in Germany. *Verticillium lecanii* was exclusively found at locations in the drinking water system where newly buried pipes had been put into service. It did not belong to the resident flora of the water derived from groundwater. *V. lecanii* probably infected the water in newly buried pipes by soil or infected arthropods before the pipes were closed. The fungus did not persist long enough to become resident flora. Van der Pas (2000) demonstrated the short persistence of *V. lecanii* in water.

RMS comments on Koppert Beheer (2004) at renewal

In this short statement (full text is shown above), two studies are cited (i.e., Göttlich et al., 2002 and Van der Pas 2000). A summary of and RMS comments on the study of Göttlich et al., 2002 is given under point B.8.3 (Effects of the microorganism on drinking water analysis. Below a summary of Van der Pas (2000) and RMS comments are provided. RMS disagrees with the statement made in Koppert Beheer (2004) that the study of Van der Pas (2000) demonstrates the short persistence of *Verticillium lecanii* in water.

Reference	Van der Pas (2000) Behaviour of *Verticillium lecanii* spores in water.
Reference number	IIM 7.1.2/03
Guideline	-
Test substance	*Lecanicillium muscarium* Ve6 (a.s. of MYCOTAL)
Previous evaluation	DAR, 2006
Source	Koppert Beheer B.V., Department R&D Microbials and Regulatory affairs (unpublished)
GLP	-

Study report ACTIVE INGREDIENT *V. lecanii*

Reference/notifier	:	Van der Pas (2000)	GLP statement	:	no
Type of study	:	germination test in water	Guideline	:	no
Year of execution	:	2000	Acceptability	:	acceptable
Test substance	:	*L. muscarium* (a.s. of MYCOTAL); batch: unknown; purity: 7.5×10^{10} spores/gram			

Material and methods:

Test concentration:	3×10^{8} spores/mL
Test system:	Erlenmeyer, 500 mL
Temperature:	21.0 ±1.0 ° C
Description:	Conidiospores of *L. muscarium* were suspended in 500 mL sterile demi water. One Erlenmeyer was kept on a magnetic stirrer constantly, the other Erlenmeyer was not stirred after preparation of the suspension
Sampling time points:	Spores were sampled daily and examined for germination
Method of analysis:	Direct germination test and indirect germination test. In the direct germination test

the sampled spores were directly examined for germination. In the indirect germination test the sampled spores were diluted and droplets from this dilution were brought onto water agar plates. The germination was assessed after 16-20 hours

Water Characteristics: Sterile demi water, pH 7.5

Methods:

Two gram conidiospores of *Verticillium lecanii* (with a concentration of approx. 7.5x10E^{10} sp/g) were suspended in 500 ml sterile demi water in a 1 liter erlenmeyer. The pH of the demi water was approx. 7.5. The spores were suspended using a magnetic stirrer, for 30-45 minutes. One erlenmeyer was kept on the magnetic stirrer constantly. The other one was taken from the magnetic stirrer so that the spores could settle to the bottom of the flask.

After certain periods of time samples were taken from both flasks. Form the non-stirred erlenmeyer only spores that were settled to the bottom were sampled. The spores were directly examined for germination (this was the so-called direct germination test). Also the spore sample was diluted 10-100 times (depending on the concentration of spores in the sample) and 30 µl droplets from this dilution were pipetted onto water agar plates. The germination of the spores on the plates was assessed after 16-20 hours (the so-called indirect germination test).

Results:

The results are shown in table 1. The spores that were kept on the magnetic stirrer permanently started to germinate after approx. 8 hours. Germination of all spores was accomplished after approx. 20 hours. Newly formed blastospores were already formed at that time. After 2 days the germination tubes (from the conidiospores) started to disappear and only the freshly formed blastospores remain in the solution. When the spores were put on water agar they were still able to germinate, even when they were stirred for 7 days. The spores in the non-stirred erlenmeyer did not geminate during the whole test period. When they were put on agar there was a clear decline in germination (35%) after 2 days with no germination at all after 6 days.

Table 8.1.2-a: Direct and indirect germination of *L. muscarium* spores in time.

Time after test start	**Direct germination (% of initial numbers of spores)**		**Indirect germination (% of initial numbers of spores)**	
	Stirred	**Not stirred**	**Stirred**	**Not stirred**
8 hours	5-10	0	-	-
1 day	>90	0	>95	>95
2 days	0*	0	>95	65
3 days	0*	0	>95	0
4 days	0*	0	>95	4
5 days	0*	0	>95	20
7 days	0*	0	>95	0

* New spores, which did not germinate, formed from initial spores

- no test carried out

Conclusion:

This research clearly indicated that spores of *Verticillium lecanii* can not germinate when they are kept in a non aerated situation. Not only they are not able to germinate directly but also loose their viability at all if they are kept too long in such a situation. Only the spores that are attached to the glass surface are able to germinate and form a mycelium mat with sporulating fungus (no data presented). The reason that still 20% germination was found in the non-stirred flask after 5 days (with the indirect germination test) was probably caused by spores from this mycelium mat when a sample from the sediment was taken. It also seems that spores are not triggered to germinate anymore when the

circumstances are not favourable or if there is a lack of nutrition's. This is probably what happened in the stirred situation The newly formed spores did not germinate as contrast with the initial spores that germinated very rapidly.

The half-life time of *V. lecanii* spores is about 3 days under sterile non aerated conditions. In a non sterile situation this might even be shorter because of competition with other micro organisms. On the other hand *V. lecanii* spores can survive for a long time in soil or in an aerated liquid situation. Therefore in the case of spores entering the surface water with the spray residues, only the spores that are on top of the surface or in an aerated or agitated situation will survive.

Remarks RMS: In this study the water temperature and oxygen concentration in the water has not been measured. It has not been proven convincingly that anaerobic conditions cause the decrease of germination, although it seems to be the most likely reason. The study should have lasted longer to determine the half-life of *L. muscarium* in stirred water.

The results that the half life in unstirred water is a little more than 2 days and in stirred aerated water spores persist for more than 95% after 7 days can be used for risk assessment.

RMS comments for the renewal on Van der Pas (2000)

The study shows that 95% of spores of *Lecanicillium muscarium* are viable after 7 days in stirred, sterile demi-water. The number of viable spores in the samples from the unstirred incubation declined during the incubation, which is attributed to a lack of aeration in the incubation. However, for the unstirred incubation samples were only taken from the bottom of the erlenmeyer, while mycelial mats were forming on the glass surface. The lack of viable spores on the bottom of the erlenmeyer could therefore also be caused by a higher attachment of the fungus to the glass surfaces in the non-stirred incubations. The fact that 95% of *Lecanicillium muscarium* Ve6 spores remain viable in sterile demi-water can be used for the risk assessment.

B.8.1.2.1 Predicted environmental concentrations in water

The predicted initial environmental densities in surface water upon application (PEDsw, initial) of the representative formulation was calculated by the RMS with the equation described below. The German Rautmann drift values were used in combination with the parameterisation of the TOXSWA-NL standard drainage ditch (i.e., 30 cm deep with a slope of 45 degrees and a volume of 210 L/m^2). For greenhouse uses of MPCA, RMS NL uses a drift percentage of 0.1%.

PEDsw, initial (CFU/L) = AR × n × (D/100) / 10.000 × Vd

- AR is application rate (CFU/ha)
- n is number of applications per year
- D drift percentage
- 100 conversion from percentage to fraction
- 10.0000 is the conversion from ha to m^2
- Vd is volume of the standard ditch per m^2

Table 8.1.2.1-1 PEDsw calculations for *Lecanicillium muscarium*

Crop	F/G*	g a.s./ha per application	CFU/ha per application	Max. number of applications	Drift** (%)	$PED_{sw, initial}$ mg a.s./L	$PED_{sw, initial}$ CFU/L
fruiting vegetables of Cucurbitaceae	G	96	2×10^{13}	12 per use; 36 per season	0.1	5.49×10^{-4} per use; 1.65×10^{-3} per season	1.14×10^{5} per use; 3.43×10^{5} per season
fruiting vegetables of Solanaceae	G	96	2×10^{13}	12	0.1	5.49×10^{-4}	1.14×10^{5}
strawberry	G	48	1×10^{13}	12 per use; 24 per season	0.1	2.74×10^{-4} per use; 5.49×10^{-4} per season	5.71×10^{4} per use; 1.14×10^{5} per season
strawberry	F	48	1×10^{13}	12 per use; 24 per season	1.52	4.11×10^{-3} per use; 8.23×10^{-3} per season	8.57×10^{5} per use; 1.71×10^{6} per season
floriculture crops, except cut roses	G	96	2×10^{13}	4 per use; 24 per season	0.1	1.83×10^{-4} per use; 1.10×10^{-3} per season	3.81×10^{4} per use; 2.29×10^{5}
cut roses	G	144	3×10^{13}	24	0.1	1.65×10^{-3}	3.43×10^{5}
tree nursery crops	G	96	2×10^{13}	24	0.1	1.10×10^{-3}	2.29×10^{5}

* F = field application; G = greenhouse application

** Ganzelmeier and Rautmann drift values according to the BBA (Federal Biological Agency of Agriculture and Forestry, Germany) 2000: Bekanntmachung des Verzeichnisses risikomindernder Anwendungsbedingungen für Nichtzielorganismen. Bundesanzeiger 100: 9878-9880.

B.8.1.3 Air

General conclusion: Spores of *L. muscarium* only are present in the air directly after an aerial application. Concentrations rapidly decrease after application. The stickiness of spores of *L. muscarium* do not favour an active aerial release. In the studies of Rainer et al. (2000) and Ulevicius et al. (2004, not submitted) the absence of spores of *Lecanicillium* spec. in the air was confirmed. It is concluded that transport of spores will not occur through the air. If spores are released in the air after an application, these spores will settle quickly. Nevertheless, spores may be transported by drift. These spores loose virulence by the solar radiation. Surviving spores will quickly degenerate in the soil, on plant surfaces or in water. The natural background concentrations of *L. muscarium* in air are not expected to be considerably raised after an aerial application.

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.

From peer-reviewed open literature no additional references were identified to be relevant for persistence of *L. muscarium* Ve6 in air. Please refer to the literature search submitted in Point MA 7.1.

RMS comments for renewal

The notifier cites the study of Rainer et al. (2000) to demonstrate that spores of *Lecanicillium muscarium* do not occur in air. This study concerns the biodiversity of airborne fungi in a hospital and in the opinion of the RMS does not provide any information regarding the persistence and multiplication of *Lecanicillium muscarium* in air. No reference or report is provided for the citation of Ulevicius et al. (2004), however the RMS assumes the notifier refers to a paper by Ulevicius et al. in Environmental Toxicology (2004) on field studies of airborne fungal propagules. Although *Lecanicillium* was not detected in this study, other entomopathogenic fungi were detected (*Beauveria*, *Paecilomyces*, and *Metarhizium*). These fungi were identified 'using macro- and micromorphological observations for the commonly used diagnostic media and consulted keys'; it is unclear to the RMS whether *Lecanicillium* spp. can be distinguished from other entomopathogenic fungi using these methods.

In a greenhouse environment the number of spores detected in air were back to background levels within a day after greenhouse application (Samson, 1990). Taken together with the global occurrence of *Lecanicillium muscarium*, it is the opinion of the RMS that the use of *Lecanicillium muscarium* Ve6 as MPCA will not result in persistent populations in air that are higher than background densities of this microorganism.

Cited studies, scientific peer-reviewed open literature and other references:

Reference	Samson (1990) Determination of fungal spores in cucumber greenhouses before and after application of Mycotal.
Reference number	IIM 7.1.3/02
Guideline	-
Test substance	*Lecanicillium muscarium* Ve6 (Mycotal)
Previous evaluation	DAR, 2006
Source	Dutch Fungal Biodiversity Institute (Centraalbureau voor Schimmelcultures, part of the Royal Netherlands Academy of Arts and Sciences)
GLP	-

Study report ACTIVE INGREDIENT *L. muscarium*

Reference/notifier	:	Samson (1990)	GLP statement	:	no
Type of study	:	determination spores in air	Guideline	:	no
Year of execution	:	1990	Acceptability	:	acceptable
Test substance	:	MYCOTAL (1.0×10^{10} spores of *L. muscarium* per gram product); batch: unknown			

Material and methods:

Test concentration:	450 g MYCOTAL, equivalent to 4.5×10^{12} spores, dissolved in 450 L water. 7.5×10^5 spores/L air when a greenhouse with a volume of 6000 m^3 is considered
Test system:	Seventeen spans (1500 m^2) of greenhouses at two locations were treated with MYCOTAL suspension

Temperature:

Sampling time points:	Samples were taken 15 minutes before and after first MYCOTAL application, 22 hours and a week after first application and on 17 April (one week after last application)
Method of analysis:	Air sampling took place at a height of 1.2 m. MYCOTAL was applied on March 27, April 4 and April 11, 1990. A two-trap Andersen air sampler and a Biotest device were used for sampling. Petri-dishes and strips (containing 2% malt agar or Dichloran glycerol 18% agar) of samplers were incubated at 25°C for 7 days

Results:

The amount of measurable fungal spores, mainly existing of *L. muscarium*, had been increased when sampled 15 minutes after application (Table 8.1.3-1). Assuming a spread of 7.5×10^5 spores per litre air during application, only a small part (i.e. less than 0.01%) of the spores is recovered from the air after 15 minutes. The amount has decreased to almost the level of before application after 22 hours. The fungi recovered then belong to the generally occurring species. Hardly any *L. muscarium* was detected in the air even after several applications with possible re-growth of *L. muscarium* on the leaves and on the whitefly larvae possibly available. In the table below the numbers of fungal colonies in the air is given. Results are only presented for the Anderson air sampler. Those of the Biotest sampler have not been given in this summary as the *L. muscarium* had not been recognisable. A small part (< 0.01%) of the sprayed spores is recovered from the air after 15 minutes. The spore concentration in the air had decreased to background level (i.e. concentration before application) after 22 hours.

Table 8.1.3-a: Number of fungal colonies – air sampling Andersen air sampler (41.5 litre)

Date		MEA*	
	Location 1	Total number of fungal colonies in air	Number of *L. muscarium* colonies in air
27-03	Before application	11	(0)
27-03	15 minutes a.a.***	428	(420)
28-03	22 hours a.a.	8	(3)
03-04	168 hours a.a.	1	(0)
17-04	504 hours a.a.	6	(4)
	Location 2		
27-03	Before application	4	(0)
27-03	15 minutes a.a.	404	(397)
28-03	22 hours a.a.	8	(0)
03-04	168 hours a.a.	4	(0)
17-04	504 hours a.a.	3	(0)

* MEA: 2% malt extract agar

Date		Dg 18**	
		Total number of fungal colonies in air	Number of *L. muscarium* colonies in air
	Location 1		
27-03	Before application	3	(0)
27-03	15 minutes a.a.***	157	(156)
28-03	22 hours a.a.	2	(0)
03-04	168 hours a.a.	5	(1)
17-04	504 hours a.a.	8	(2)
	Location 2		
27-03	Before application	2	(0)
27-03	15 minutes a.a.	1112	(1094)
28-03	22 hours a.a.	2	(0)
03-04	168 hours a.a.	6	(0)
17-04	504 hours a.a.	7	(0)

** DG 18: Dichloran 18% glycerol agar

*** a.a.: after application

Remarks RMS: Numbers without the brackets represent the total number of fungal colonies, including all species. Other species included *Penicillium*, *Aspergillus* and *Botrytis cinerea.* These species were especially noted in the Biotest strips. The numbers of colonies of other species remained stable throughout the experiment, indicating that the determination of *V. lecanii* was accurate.

RMS comments on Samson (1990) at renewal

No comments.

B.8.2 Mobility

Statement

The applicant states that the mechanisms of spread of *L. muscarium* are not exactly known. It has been speculated that aphids carry spores from the soil to the leaves, causing infection in other insects. Spores are not spread by air, naturally, and are not released from conidiophores without water contact. Conidia artificially released by aerial applications, however, have a short life span after drying up, preventing spread of infection by the air (Gardner et al., 1984). Passive spread can occur by means of splashing, and probably by mechanic transfer by other Arthropoda present in the greenhouse (Rombach and Gillespie, 1988). In surface water the spores will quickly sediment and loose their viability. Therefore mobility of *L. muscarium* after applications is not expected to occur.

General conclusion:

The mechanisms of spread of *L. muscarium* are not exactly known. Aphids may carry spores from the soil to the leaves, causing infection in other insects. Spores are not spread by air, naturally, and are not released from conid-

iophores without water contact. Passive spread can occur by means of splashing, and probably by mechanic transfer by other Arthropoda present in the greenhouse. Mobility of spores through leaching to the groundwater does not occur.

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.

From peer-reviewed open literature no additional references were identified to be relevant for mobility of *L. muscarium* Ve6. Please refer to the literature search submitted in Point MA 7.1.

RMS comments on mobility at renewal

In three soils tested (sand, loamy sand, sandy loam), no leaching of *Lecanicillium muscarium* Ve6 was detected (Hollingsworth, 1983). Long-range transport through air is not expected to be an important factor in the dispersal of *Lecanicillium muscarium* Ve6, as numbers of spores detected in air are back to background levels within a day after greenhouse application (Samson, 1990).

Short-range dispersal of *Lecanicillium muscarium* is likely to occur through rain-splash. In addition, dispersal can occur by arthropods (Down et al., 2009).

Cited studies, scientific peer-reviewed open literature and other references:

Reference	Hollingsworth (1983) *Verticillium lecanii* degradation and percolation in soil.
Reference number	IIM 7.1.1/04
Guideline	Merkblatt No. 55 Parts I and II of the German Federal Biological Institute for Agriculture and Forestry
Test substance	*Lecanicillium muscarium* Ve6
Previous evaluation	DAR, 2006
Source	Tate & Lyle PLC, Group Research and Development (unpublished)
GLP	-

Study report ACTIVE INGREDIENT *L. muscarium*

Reference/notifier	:	Hollingsworth (1983)	GLP statement	:	no
Type of study	:	percolation in soil	Guideline	:	B.B.A. Merkblatt No. 37
Year of execution	:	1983	Acceptability	:	acceptable
Test substance	:	*L. muscarium* (1.9×10^{10} spores of per gram); Batch: VE6-57			

Percolation:

Test concentration:	1 mg aliquots of MYCOTAL equivalent to 10^7 CFU *L. muscarium*/column
Test system:	Tubes measuring 35 cm in height × 5 cm in diameter, filled with soil to a depth of 30 cm
Temperature:	Low room temperatures to minimise evaporation
Sampling time points:	At days 7, 14, 21 and 28

Method of analysis:	1 gram soil was added to 9 ml solution. A dilution series was prepared. 0.1 ml aliquots of each dilution were plated on selective medium (rose Bengal choramphenicol agar). Plates were incubated at ambient laboratory temperature, 22 ± 2 ºC. Plates were examined after 3, 5 and 7 days recording the number of V. lecanii colonies and calculating the total number per g soil.
Soil Characteristics:	Standard Speyer 2.1 soil: low humus, sand; %OC 0.66; pH 6.5; MWHC 12% Standard Speyer 2.2 soil: high humus, loamy sand; %OC 2.9; pH 6.1; MWHC 20% Standard Speyer 2.3 soil: medium humus, sandy loam; %OC 1.16; pH 6.0; MWHC 24%.
Test material:	MYCOTAL
Description:	Glass percolation tubes were filled with washed soils (soils 2.1, 2.2 and 2.3) to a depth of 30 cm. Soil columns were pre-saturated with water. The water flow was adjusted to 10-12 mL/hr (leaching by ≈200 mm rainfall). One mg (± 1×10^7 CFU/column) was added on top of the test soil. Filtrate was collected and sampled

Results:

In Table 8.2.a the results on soil percolation are given.

Table 8.2.a Soil percolation of *V. lecanii* strain Ve6 in soil at ambient temperature (1×10^7 CFU/column added).

Soil type1	**Number of propagules recovered from soil1**									
	Day 4		**Day 7**		**Day 14**		**Day 21**		**Day 28**	
	B/Y	**M**	**B/Y**	**M**	**B/Y**	**M**	**B/Y**	**M**	**B/Y**	**M**
sand	4.4×10^3	2.0×10^2	1.2×10^3	6.0×10^2	3.0×10^3	10	3.5×10^3	25	1.6×10^3	2.5×10^2
loamy sand	9.0×10^2	10	2.5×10^3	4.5×10^2	4.5×10^3	1.5×10^3	6.5×10^3	15	1.5×10^4	1.5×10^3
sandy loam	1.0×10^4	25	1.4×10^3	2.5×10^2	6.5×10^2	10	7.5×10^2	10	2.2×10^3	40

1 B/Y: Bacteria and yeasts; M: Filamentous moulds (not *L. muscarium*).

After four days there was no evidence of *L. muscarium* present in the filtrates. Continued percolation until 28 days did not reveal the presence of *L. muscarium* in the filtrates either.

Conclusions: *L. muscarium* does not leach through soil into ground water.

RMS comments on study Hollingsworth (1983) for renewal:

No comments on the summary provided above. Note that this study was also submitted under point B.8.1.1 (Persistence and multiplication in soil), where a summary of this study regarding degradation of *Lecanicillium muscarium* in soil is given.

Other studies on mobility:

Reference	Rombach & Gillespie (1988) Entomogenous Hyphomycetes for insect and mite control on greenhouse crops
Title	IIM 7.1.1/06
Guideline	-
Test substance	-

Previous evaluation	DAR, 2006
Source	Biocontrol News & Information **9**(1): 7-18 (published)
GLP	-

Study 1

In a review by Rombach and Gillespie (1988) it is stated that the mode of transmission of the infective particles, from soil to the upper parts of the plant and subsequently to the scales or aphids, is not known. Active, live aphids carrying saprophytically growing and sporulating mycelium were observed and it was suggested that these might well be an important factor in the distribution of the fungus within the pest population (references in Rombach, 1988). Conidia of *V. lecanii* are dispersed only after wetting, but the dislodged conidium has a short half-life following drying and therefore the chances of airborne infections are remote (reference in Rombach, 1988).

Hyphal bodies in the commercially formulated products of *V. lecanii* can survive on the foliage for considerable periods of time at favourable humidity. Dispersal of inoculum from its source to the insects and between infected and healthy insects by overhead watering or by non-host insects and mites has also been suggested (reference in Rombach, 1988).

RMS comments on Rombach and Gillespie (1988) at renewal

No comments.

Reference	Hall (1981) Laboratory studies on the effects of fungicides, acaricides and insecticides on the entomopathogenic fungus, Verticillium lecanii.
Reference number	IIM 7.1.1/02
Guideline	-
Test substance	*Verticillium lecanii*
Previous evaluation	DAR, 2006
Source	Entomologia Experimentalis et Applicata **29**: 39-48 (published)
GLP	-

Study 2

The study of Hall (1981) deals with the compatibility of *V. lecanii* with chemical fungicides, acaricides and insecticides. Since dispersal of *V. lecanii* was not the issue of the publication, no information can be used for the risk assessment.

RMS comments on Hall (1981) at renewal

No comments on the study. Note that this study was also submitted under point B.8.1.1 (Persistence and multiplication in soil).

Reference	Down et al. (2009) Dissemination of the entomopathogenic fungi, *Lecanicillium longisporium* and *L. muscarium*, by the predatory bug, *Orius laevigatus*, to provide concurrent control of *Myzus persicae*, *Frankliniella occidentalis* and *Bemisia tabaci.*
Reference number	IIM 7.2
Guideline	-
Test substance	*Lecanicillium muscarium*
Previous evaluation	Submitted by RMS for the purpose of renewal
Source	Biological control **50** 172 – 178 (published)
GLP	-

Abstract

The simultaneous use of two biocontrol agents for the concurrent control of three pest species was investigated. Leaf disc bioassays were conducted to establish a suitable method (surface dosing) for the dissemination of an entomopathogenic fungus (*Lecanicillium longisporum* or *L. muscarium*) by the predatory bug *Orius laevigatus*. Predatory bugs surface dosed with fungal conidia successfully disseminated conidia onto sweet pepper leaf discs. Most (98%) of the peach-potato aphids (*Myzus persicae*) that were subsequently maintained on the leaf discs became infected with the pathogen and died within 5 days. However, fungal conidia disseminated by surface dosed predatory bugs did not infect and kill the western flower thrips (*Frankliniella occidentalis*) or the sweet-potato whitefly (*Bemisia tabaci*). Plant trials were performed to assess the efficacy of using predatory bugs surface dosed with *L. longisporum* as an effective means of controlling *M. persicae* and *F. occidentalis* populations. The results indicated that the number of aphids and thrips were significantly lower (66% and 95%, respectively) on the plants where surface dosed predatory bugs were used as a control measure compared with plants where the fungal pathogen alone was used and were statistically comparable to the numbers on plants where the predatory bug alone was used. The potential for using this dual approach is discussed in the context of improved biological control of glasshouse pests.

RMS comments on Down et al., 2009

The ability of a predatory bug (*Orius laevigatus*) to disseminate *Lecanicillium muscarium* Ve6 was determined. Adult predatory bugs were dosed with *Lecanicillium muscarium* Ve6 by being confined in a box in which a 'fungal lawn' was present that consisted of a culture of *Lecanicillium muscarium* Ve6 on agar plates. Predatory bugs used as experimental control were not exposed to *Lecanicillium muscarium* Ve6. The predatory bugs were subsequently confined in culture plates in which leaf discs of sweet pepper plants were present. After 48 hours the leaf discs were transferred to glass tubes and 10 newly mature aphids (*Myzus persicae*; peach-potato aphids) were placed on the discs. The survival of the aphids was monitored. Ninety-eight percent of the aphids kept on leaf discs that were exposed to predatory bugs dosed with *Lecanicillium muscarium* Ve6 became infected with the fungal pathogen, versus 4% of the aphids on leaf discs exposed to the control predatory bugs. This study demonstrates that dispersal of *Lecanicillium muscarium* Ve6 can occur through arthropod vectors.

B.8.3 Effects of the microorganism on drinking water analysis

New data 2016

Municipal treatment of drinking water is anticipated to reduce any potential risk to adults, children, and infants. The species *L. muscarium* does not produce any toxins or secondary metabolites of toxicological concern at substantial quantities and therefore leaching to groundwater is not relevant to this fungus. Interference with analytical methods for drinking water will not occur since methods used for the identification of drinking water contaminants listed under Directive 98/83/EC use specific media coupled to dye reactions. As contaminants that are monitored are not related to *L. muscarium,* it will not grow on these media and consequently not influence the methods.

RMS comments on effects of the micro-organism on drinking water analysis for renewal

In three soils tested (sand, loamy sand, sandy loam), no leaching of *Lecanicillium muscarium* Ve6 was detected (Hollingsworth, 1983). During a 12-month survey on groundwater-derived drinking water from 29 water supplies in Germany, *Verticillium lecanii* was detected only exclusively at locations with newly buried pipes. *Verticillium lecanii* is therefore not considered to be a member of the permanent fungal flora of drinking water systems. Exposure of surface water to *Lecanicillium muscarium* Ve6 as a result of the proposed use is possible due to for example spray drift and dispersal by arthropods.

Lecanicillium muscarium does not produce any metabolites of toxicological concern (see section B.2.7) and it is unlikely that this MPCA will grow on the selective media that are used to monitor drinking water quality, therefore no interference with the analytical systems for the control of the quality of drinking water are expected.

No interference of the microorganism with the quality control analyses of the product Mycotal were observed; these analyses include the determination of the content of microbial contaminants (see Volume 4 Section C.1.3.4).

Cited studies, scientific peer-reviewed open literature and other references:

Lecanicillium muscarium final addendum November 2009: Open point: 8.5

RMS to evaluate the study Gottlich et al 2002 in an addendum, if the study was present in the notifiers' dossier.

Reference	Göttlich (2002) Fungal flora in groundwater-derived public drinking water.
Reference number	IIM 7.1.2/01
Guideline	-
Test substance	-
Previous evaluation	DAR, 2006
Source	International Journal of Hygiene and Environmental Health **205**: 269-279 (published)
GLP	-

Study summary

In a 12-month survey on groundwater derived drinking water from 29 water supplies in Germany, the possible dissemination of fungi via the public drinking water distribution system was assessed. Results were obtained by long term incubation of 1 ml aliquots of water samples on agar-based culture media. Verticillium lecanii was exclusively found after the introduction of newly buried pipes and remained localised at those sites. Introduction via arthropod vectors is likely.

Methods of identification

Isolation of fungi was performed using 1 ml water sample plated on culture plates with blood agar base (Oxoid) incubated at 20 ºC for up to 4 weeks and examined weekly. Fugal colonies were enumerated and transferred to fresh potato dextrose agar and malt extract agar and maintained at 20 ºC. Whenever possible filamenteous fungi were phenotypically identified down to species level by morphology. The morphological identity of a limited number of representative strains were confirmed at the Centraalbureau voor schimmelcultures.

Results

Verticillium was detected in 7 out of a total number of 670 water samples from locations with newly laid pipes. It was detected in 1 raw water sample out of 511 raw water samples.

Results from sampling locations with newly buried pipes are considered not representative of long-term water quality.

Conclusion

Verticillium lecanii was found nearly exclusively at locations with newly buried pipes, thus this species is considered to belong to a transient fungal flora with localised distribution. For V.lecanii introduction in the water network via soil and arthropod vectors is considered likely.

RMS comments on Göttlich (2002) at renewal

No comments

B.8.4 References relied on

Data point	Author(s)	Year	Title Owner Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner
IIM 7/01	Fenice M, Gooday GW	2006	Mycoparasitic actions against fungi and oomycetes by a strain (CCFEE 5003) of the fungus *Lecanicillium muscarium* isolated in Continental Antarctica Annals of Microbiology **56**(1) 1-6 GLP: no published				
IIM 7.1/01	Beyer PU Hirte WF Sermann H	1997a	The behaviour of the entomopathogenic fungus *Verticillium lecanii* (Zimm.) Viegas in soil: II. Longevity of *V. lecanii* in soil and mineral wood and the optimization of its survival by addition of promoting organic substances. Humboldt-Unversität zu Berlin, Inst. f. Biologie, Invalidenstr. 42, D-10115 Berlin, Germany, FG Phytomedicin/Angewandte Entomologie, Dorfstr. 9, D-13051 Berlin, Germany. Zeitschrift fuer Planzenkrankheiten und Planzenschutz, 104(1): 65-74. GLP: no published	N	N		-

Data point	Author(s)	Year	Title Owner Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner
IIM 7.1/02	Beyer PU Hirte WF Sermann H	1997 b	The behaviour of the entomopathogenic fungus *Verticillium lecanii* (Zimm.) Viegas in soil: I. Viability in soil at different ecological conditions. Humboldt-Unversität zu Berlin, Inst. f. Biologie, Invalidenstr. 42, D-10115 Berlin, Germany, FG Phytomedicin/Angewandte Entomologie, Dorfstr. 9, D-13051 Berlin, Germany. Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz no. 104(1): 54-64 GLP: no published	N	N		-
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Data point	Author(s)	Year	Title Owner Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner
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IIM 7.1/07	Lysek H Fassatiova O Lopez N	1986	Autodehelminithizing capacity of soils in two Mexican localities. Department of Biology, Medical Faculty, Palaky University, Olomouc, Czechoslovakia, Department of Cryptogamic Botany, Faculty of Natural Sciences, Charles University Prague, National University, U.N.A.M., Mexico City, Mexico Helminthologia, 23(4): 237-241. GLP: no published	N	N		-
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Data point	Author(s)	Year	Title Owner Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner
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IIM 7.1/12	Zare R, Gams W	2001	A revision of *Verticillium* sect. Prostrata. IV. The genera *Lecanicillium* and *Simplicillium* gen. nov. Nova Hedwigia 73:1–50 GLP: no published	N	N		-

Data point	Author(s)	Year	Title Owner Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner
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IIM 7.1.1/05	Koppert Beheer B.V	2004	Mobility of *Verticillium lecanii.* Koppert Beheer B.V., Department R&D Microbials and Regulatory affairs, P.O. Box 155, 2650 AD Berkel en Rodenrijs, The Netherlands. Koppert Beheer B.V. Unpublished statement	N	Y		KBS

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