Renewal Assessment Report

Lecanicillium muscarium Ve6

Volume 3MA – B.7 Residues in or on treated products, food and feed

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Rapporteur Member State: The Netherlands

Co-Rapporteur Member State: France

Version history

When	What
January 2018	Initial RAR

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B Summary of the data and information

B.7 Residues in or on treated products, food and feed

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

The company Koppert B.V. is submitting a dossier for the re-approval of the microorganism *Lecanicillium muscarium* Ve6 (19-97), further referred to as *Lecanicillium muscarium* Ve6, as an active ingredient under regulation (EC) 1107/2009.
The Microbial Pest Control Agent *Lecanicillium muscarium* Ve6 (formerly *Verticillium lecanii* Ve6) was included in Annex I of Directive 91/414/EEC on 1 May 2009 pursuant to Article 24b of the Regulation (EC) No 2229/2004, (Commission Directive 2008/113/EC) and then approved according to the Commission Implementing Regulation (EU) No 540/2011 of 25 May 2011, implementing Regulation (EC) No 1107/2009 of the European Parliament. *L. muscarium* Ve6 was notified and defended by Koppert Beheer B.V. (Koppert B.V. is a 100% daughter company of Koppert Beheer B.V.). The active ingredient has been evaluated in The Netherlands according to Uniform Principles. The representative formulated product for the initial evaluation was the product MYCOTAL, containing 1.0×10^{10} spores/g.
The microorganism has been previously classified as *Verticillium muscarium.* The strain has been reclassified in 2001 as *Lecanicillium muscarium*, based on molecular analysis as RFLP and ITS sequence analysis by Zare & Gams. The taxonomy change was already considered for the peer review of the pesticide risk assessment of the active substance by EFSA[1].
Here the data is presented that were previously evaluated by RMS The Netherlands in the DAR (June 2007) and DAR addenda (June 2009, October 2009), as well as new data and information based on literature searches and studies. Previously submitted information (consolidated from DAR and addenda) is highlighted in grey, and information on the original DAR Points and the respective EU Points is complemented where necessary.

B.7.1 Persistence and likelihood of multiplication in or on crops, feedingstuffs or foodstuffs

Information from the original DAR

The intended uses of Mycotal envisaged are in cucumber, tomato, sweet pepper, strawberry and ornamentals. Mycotal is applied as spray application and is sprayed onto the undersides of the leaves and

[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

to the growing points. For good control of the whitefly population it is recommended to apply Mycotal two till four times with an interval of seven days. In case new infections occur later in the season, full treatment with Mycotal (two till four applications per treatment) can be repeated. Mycotal does not have a pre-harvest interval. However, according to good agricultural practise the product should not be sprayed on the crop on the day of harvest before harvesting.

The generation of residue data is not required for application in ornamentals since they are not used for consumption.

The notifier addresses the various aspects concerning residues in un-published documents (that are submitted to scientific journals), and some open literature that are cross-references to other articles. Although the studies described in the articles were not performed under GLP conditions, the main studies were performed as part of the RAFBCA-project (see also http://www.rafbca.com/index.asp). RAFBCA is funded under the Fifth Framework Programme of the European Commission, Quality of Life and Management of Living Resources Programme (QoL), Key Action 1 - Food, Nutrition and Health. This EC-project's general objective is the identification of metabolites, produced by fungal biological control agents (BCAs), and to establish, whether they enter the food chain and if they pose a risk to human and animal health. This project will generate data that could help address key registration questions.

New data

Lecanicillium muscarium is not known to colonize food or feed. However, some studies describe the endophytic colonization of plants by *Lecanicillium lecanii*. In a recent review by Vidal & Jaber (2015), entomopathogenic fungi are described also to grow sometimes endophytically. This is mostly described for the species *Beauveria bassiana*, whereas studies on the endophytic colonization by *L. lecanii* are reported sporadically. Moreover, it should be considered that the species *L. lecanii* clearly differ in its taxonomy to *L. muscarium*, and findings on *L. lecanii* are not necessarily transferrable to *L. muscarium.* Nevertheless, the following reports are presented, since both species are members of the genus *Lecanicillium*.

In a study with *L. lecanii* on cotton and the aphid *Aphis gossypii*, it was shown that *L. lecanii* has the ability to grow in cotton leaves, as well as to grow on the aphids (Anderson et al., 2007). *L. lecanii* was isolated more frequently from leaves damaged before inoculation than from undamaged leaves. After 35 days, *L. lecanii* was isolated from 100% of damaged leaves, but much less frequently from undamaged leaves. However, no detailed information is presented in the reports. Moreover, due to the

study design, results are not necessarily transferrable to natural conditions. Due to the lack of information results cannot be clearly evaluated and should be treated with care.

However, these endophytic colonization by *L. lecanii* is confirmed in a study by Gurulingappa et al. (2010), studying the colonization of beans, corn, wheat, tomato and pumpkin by *L. lecanii*, *B. bassiana* and *Aspergillus parasiticus*. Leaves were inoculated by spraying the leaves at a fungal population of 1×10^8 CFU/mL at the three true leaf-stage. Colonization of leaves by *L. lecanii* was highly variable between crop species. Moreover, *L. lecanii* showed a significant decline in percentage of colonization in bean. After 20 days, no living cells of *L. lecanii* were isolated from cotton leaves, stem or root. Additionally, the authors observed no colonization of plants via infested soil.

Although endophytic colonization is described for some *Lecanicillium* species and strains, it is not described for *L. muscarium* or the strain Ve6. Endophytic growth is a common phenomenon: many fungal species are capable of endophytic growth and all plants host fungal endophytes (up to 60 taxa reported for a single plant species). Moreover, plant defense systems guarantee a controlled fungal growth and equilibrated endophytism. Since *L. muscarium* Ve6 was shown not to be toxic, nor plant pathogenic or infective to mammals, and does not produce relevant metabolites, no risk would be expected due to an eventual endophytic growth on plants.

Cited references (abstracts):

KMA 6.1/02 – Vidal, S., Jaber, L.R (2015), Entomopathogenic fungi as endophytes: plant-endophyte-herbivore interactions and prospects for use in biological control;Current Science, 109: 46-54.

Abstract: It is now evident that entomopathogenic fungi are able to colonize plant tissues as symptomless endophytes. Although most data so far published in this regard refer to *Beauveria bassiana* as an endophytic fungus, two other entomopathogenic fungi, viz. *Metarhizium anisopliae* and *Lecanicillium lecanii* have also been shown to colonize plant tissues endophytically. Several recent studies have also shown reasonable detrimental effects on herbivorous insects feeding on plants harbouring these fungi as endophytes. However, data published so far are highly variable and not consistent with regard to the underlying mechanisms which would allow explaining these effects. Growth conditions, specific cultivar features, or interactions with other microorganisms may impact the effect of these endophytic entomopathogenic fungi on the herbivorous insects. Furthermore, other fungi may block the systemic growth of the fungi in plant parts distant to the point of inoculation. Other parameters which need to be taken into account for using these fungi as biocontrol agents are the level of mycotoxins produced in plants, the level of pest reduction and the nature of formulations allowing a consistent colonization of the crop plants. This review discusses these and other problems related to the use of entomopathogenic fungi as endophytic biocontrol agents.

KMA 6.1/03 – Anderson, C.M.T., McGee, P.A., Nehl, D.B., Mensah, R.K. (2007), The fungus *Lecanicillium lecanii* colonises the plant *Gossypium hirsutum* and the aphid *Aphis gossypii;* Australasian Mycologist, 26: 65-70

Abstract:

The purpose of the research reported here was to determine whether the fungus *L. lecanii*, which was isolated as an endophyte from cotton (*Gossypium hirsutum*), may readily colonise cotton, an aphid pest of cotton (*Aphis gossypii*), and transfer from plant to aphid, and from aphid to plant. *L. lecanii* from agar culture and growing on or in alternative hosts was used to inoculate the aphid *A. gossypii* and cotton *G. hirsutum.* Colonisation was assessed by isolating the fungus from the surface or from within each host. *L. lecanii* colonised each host from spores, and transferred from aphid to cotton and cotton to aphid. The fungus sporulated on the surface of both hosts. Internal colonisation of each host increased over time under certain conditions. In conclusion, the entomopathogen *L. lecanii* readily colonised two potential hosts and transferred between the hosts under experimental conditions. *L. lecanii* is widely used in glasshouses to control aphids. The potential for the entomopathogen to survive in field conditions is indicated by these results. If the outcome is supported under field conditions, the fungus may be used to reduce the impact of a pest of cotton in Australia.

KMA 6.1/04 – Gurulingappa, P., Sword, G.A., Murdoch, G., McGee, P.A. (2010), Colonization of crop plants by fungal entomopathogens and their effects on two insect pests when in planta; Biological Control, 55: 34-41.

Abstract: Fungal entomopathogens can directly regulate populations of various insects. The entomopathogen *Beauveria bassiana* can also endophytically colonize various plants. Endophytic colonization by entomopathogens might be more widespread than currently realized and may provide a source of indirect interactions between fungi and insects. We tested whether some common entomopathogens could colonize six crop plants. We also assessed whether the performance of two insects, *Aphis gossypii* and *Chortoicetes terminifera*, was affected by entomopathogens in plants. The entomopathogens *B. bassiana*, *Lecanicillium lecanii* and *Aspergillus parasiticus* individually colonized the leaves of all six crop plants when inoculated as conidia. *L. lecanii* also readily colonized five different cultivars of cotton. When the entomopathogens were present in the soil in which either cotton or wheat seedlings were grown, *A. parasiticus* was subsequently isolated from the leaves, stem and roots of both plants and *B. bassiana* from the leaves, stem and root of wheat only, whereas *L. lecanii* failed to colonize either plant through the soil. Of the three entomopathogens tested, endophytic presence of *A. parasiticus* reduced growth of cotton, but none reduced growth of wheat. Feeding by *A. gossypii* on cotton leaves colonized by either *B. bassiana* or *L. lecanii* slowed aphid reproduction, and consumption of wheat leaves colonized by either *B. bassiana* or *A. parasiticus* slowed the growth of *C. terminifera* nymphs. The life cycle of at least three entomopathogens potentially includes plants. The presence of entomopathogens as endophytes can influence growth and fecundity of insect herbivores, suggesting a possible role for endophytic entomopathogens in the regulation of insect populations.

B.7.2 Further Information required - Exposure to consumers

B.7.2.1 Non-viable residues

Information from the original DAR

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. Moreover, in the RAFBCA project the presence of toxins in the formulation, spores, and food crops was analysed (Skrobek et al., 2005 submitted); see B.7.2.1 for a detailed evaluation of this study. The general conclusion was that no destruxins, considered to be the important tox-

ins produced by *L. muscarium* in laboratory cultures, could be detected in the product Mycotal, in the unformulated spores or in tomatoes or cucumbers after application of Mycotal (10 times the recommended dose).

Concluding it can be said that dtxs - considered to be the most important toxins for *V. lecanii* Ve6 - were not produced by Ve6 when produced under conditions as practised in commercial situation (solid state fermentation and in aerated (shaken) liquid fermentation). Moreover, the mode of action of *V. lecanii* Ve6 is not considered to be based on toxins. It can therefore be concluded that no further residue data on the metabolites (e.g. persistence, stability) are required.

However, as the RAFBCA is an elaborate project, the behaviour of dxts in the environment was investigated. The RMS considers these results to be of general interest for the understanding of the relevance and behaviour of fungitoxins in the environment, and hence summaries and conclusions of RAFBCA are presented below.

In the final report of partner 01 in the RAFBCA-project (Butt et al., (2004), already referred to in B.2.1.8.) the stability and persistence of the purified metabolites destruxins A, B and E was assessed under various environmental conditions. The effects of humidity, temperature and UV light were tested. Degradation under greenhouse conditions was assessed on object slides as well as on tomato plants. In addition, the effect of boiling and microwaving was investigated. It should be noted that these are all worst-case scenarios compared to the normal practice of spraying Mycotal, since the purified dtxs were applied in these studies.

The stability of the metabolites during storage - dry and greenhouse conditions - was temperature-dependent; half-lives decreased with higher temperatures. Half-lives were 9.6, 7.7, and 1.9 months at 25ºC for destruxin A, B and E, respectively under dry storage conditions. These half-lives were similar to those found under greenhouse conditions.

Relative humidity and UVA and UVB had little or no effect on the storage stability of the destruxins A and B; Dtx E could not be recovered after 28 days of storage at humidities below 90%. Degradation of destruxins on objects slides under greenhouse conditions was low; destruxin E decreased with 20% 7 and 14 days after application, whereas destruxins A and B were stable. Degradation on tomato leaves was also studied. Seven days after application, destruxins A and B were slightly decreased (but not significantly), and destruxin E was decreased with approximately 40%. After 14 days, the three destruxin were decreased to 40, 20, and 26%, for destruxin A, B and E, respectively.

Exposure of the destruxins to 100ºC resulted in the recovery of about 70% of the destruxins A and B after 43 minutes. Destruxin E was undetectable after 13 minutes.

From the above it can be concluded that purified destruxins A, B and E applied as such were still present on tomato leaves after two weeks under greenhouse conditions (20-40% of the applied amount). However, according to Skrobek et al. (2005 submitted) destruxins were not detected in the fruits after a foliar application to the plants, nor in the extract of the product Mycotal. There is a (theoretical) possibility that *V. lecanii* produces destruxins inside the host insect, however this is not known, and in that case it will be in extremely low quantities, and will not be recovered from the leaves or fruits. Moreover, it should be noted that in the above-described experiments with purified toxins, there was no interaction with other compounds possible, e.g. no breakdown by enzymes, organic acids, etc, and the test were performed on inert substrate (glass), hence detoxification by the plant itself could not have occurred.

Conclusion

With regard to the persistence and stability of metabolites on (edible parts of) crops, it can be concluded that the destruxins itself, when applied as such, were still present on tomato leaves after two weeks under greenhouse conditions. However, no toxins are expected to occur during and after application of Mycotal.

Exposure to consumers A document was submitted that concerns a study on the production of destruxins by Mycotal (Skrobek, et al. (2005 submitted), submitted to Food and Chemical Toxicology); the study was part of the RAFBCA project. Tomato and cucumber plants received a foliar application of Mycotal at a dose of 10^8 spores/mL of spray (= 2×10^{14} spores/ha, i.e. 10 times the recommended dose). The tomato plants were treated 5 months after sowing, whereas the cucumber plants received a treatment 2 months after sowing. Mycotal was applied 3 times with an interval of 1 week. Fruits were sampled one day after the first application and one day after the third application (= 15 days after the first application). After harvesting, fruits were stored at -18ºC until analysis. For analysis, fruits were homogenised in TRIS buffer and pre-purified on Waters' C18 environmental cartridges using acetonitrile (leaves were not subjected to extraction and analysis). Extracts were re-dissolved in a methanol/acetonitrile-mixture. Additionally, tomato fruits were homogenised in a blender and extracted with a mixture of dichloromethane and ethyl acetate. To study the presence of metabolites in the product, both Mycotal and its unformulated spores were separately crushed in liquid nitrogen and extracted with dichloromethane. The polar and non-polar fractions were subjected to analysis.

Analysis of extracts was performed by HPLC using known standards for identification. Standards used were destruxins A, B and E. The cytotoxicity of the extracts was tested using the insect cell line Sf-9.

In the polar and non-polar extracts of the product Mycotal and its unformulated spores, destruxins A, B and E could not be detected. Some extracts exhibited a few peaks in the HPLC chromatograms, which were not identified. In the Sf-9 cell line the polar extracts of Mycotal exhibited weak cytotoxic activity at 500 ppm and the cell proliferation was reduced to 86.3% but differences to the control were not significant. The unformulated spores exhibited significant cytotoxic effects on the Sf-9 cells, and cell proliferation rate was reduced to 18.2% after 3 days, but alterations in cell morphology differed from those observed for destruxins. Polar and non-polar extract from Mycotal and unformulated spores had no effects on the lepidopteran *Galleria mellonella* when injected at 1000 ppm: the treated insects did not show remarkable external signs.

Destruxins A, B and E were not detected in the extracts of the fruits of the foliar treated plants. Chromatograms of the polar extracts from tomatoes and cucumbers from the Mycotal treated plants showed distinct unidentified peaks in the chromatogram. Polar extracts from the cucumber fruit caused a decrease in cell proliferation of about 25% compared with control cell. Cell morphology was different from these cells compared to morphology of cells that were treated with destruxins (Skrobek, et al., (2005 submitted), not yet published).

Discussion

Skrobek et al. (2005 submitted) discovered that some extracts from Mycotal, unformulated spores and tomatoes and cucumber after foliar treatment contained unidentified compounds that were cytotoxic in the insect Sf-9 cell line. Destruxins could not be detected in the extracts. The amounts found are very low, and since the mode of action of V. lecanii is not considered to be based on toxins, the relevance of these unidentified compound is questioned. As is generally known, all living organisms, including *Lecanicillium muscarium* produce secondary metabolites, and with improvement of the analytical methods, more and more metabolites will be discovered. It should be taken into account that the plant protection action of Mycotal is not due to residual effects of these metabolites, no significant residues of toxins/metabolites are expected, (related) species are not known to produce toxins, and no harmful effects were observed in the toxicological studies; hence investigations into the possible occurrence of any metabolite produced by *V. lecanii* Ve6 should be considered irrelevant for the risk assessment.

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.

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

As stated in the original evaluation not very clearly is that the study showed that only low levels of metabolites were produced in insects which rapidly decline after infection and that destruxins were only produced during the infection process of the fungus and that it will be restricted in the host. Moreover, the authors assumed that the level of destruxins declined rapidly after insect death due to detoxification processes presumably via enzymes from the pathogen and lysed insect cells.

In the submitted/not published study by Skrobek et al. 2005 the toxicity of the pure metabolites and of the crude extract preparations was determined in the different test systems covering the spectrum from prokaryotes to highly developed eukaryotes as insect cells and human leucemic cells, bacteria or protozoa.

Considering the effect was found on insect cell lines and not on human cell lines together with the absence of any mutagenic or genotoxic effect or any other toxicological effect by *L. muscarium* Ve6 RMS is on the opinion that destruxin is toxicologically not relevant and no significant residues of toxicologically relevant toxins/metabolites are expected after the protection action of MYCOTAL.

In addition Lecanicillium produced destruxins formerly reported for Metarhizium (Butt et al., 2009). Studies to date showed these compounds to have activity but did not pose a risk partly because they were not detected in the food chain, were unstable and were produced such extremely small quantities (Skrobek et al., 2005, 2009; Dudley et al., 2004; Kouvelis et al 2011). A range of assays were used to determine the risk of destruxins and other bioactive metabolites (Skrobek et al., 2005, 2006; Kouvelis et al., 2011)

B.7.2.2 Viable residues

Information from the original DAR

The notifier refers to the very limited study of MacQueen & Quinlan already described in B.2.1.5. In this study Mycotal was applied to fruiting tomato in peat-based grow bags. Samples of fruit or leaves were taken after the application of Mycotal; at day 0, 1, 2, 3, 4, 11, and 12. Leaves and fruits were washed and dilutions of the wash were plated onto malt extract agar and incubated for 5 days. Numbers of colonies washed from the leaves increased significantly after 4-12 days of application (up to a 30 fold increase). The number of colonies on fruit declined to 40% of the original count (MacQueen & Quinlan, date unknown, source unknown 19XX a). However, this study is not very representative for a commercial application; the tomato plants contained more than 300 whiteflies per plant and sooty mold damage was becoming serious. Under practical conditions this level of whitefly infestation is unacceptable for a grower and they will never let it come to this level. If a few adult whiteflies per plant (less than 5) are present, a grower will intervene to reduce this number. In this study the high level of whitefly caused a lot of honeydew (excreted sugars) on the leaves, on which fungi start to grow, including *Verticillium lecanii*. This explains why there was an increase of *V. lecanii* CFUs after

10-12 days on the leaves. This represents an unrealistic scenario that will never happen in practice. On the fruits – where no whiteflies are present - there is no increase in the number of CFUs, but rather a decrease to a very low level.

Koike et al. (2004) investigated the ability of *V. lecanii* strains to colonize leaf surfaces. He demonstrated that some strains can grow epiphytically on leave surfaces, but that the Mycotal and Vertalec strains quickly decrease in numbers on leaves of various plants. After four weeks only 30-60 CFUs/mL (spores) were found back on cucumber resp. tomato leaves (less than 0.01%) and none on strawberry leaves, while 10^7 spores/mL were applied, generally resulting in around 10^5 spores/mL (application under commercial conditions is 0.1 L/m^2 equals 0.01 mL/cm^2 equals 10^5 spores/cm^2). This indicates that spore numbers decrease quickly over time.

The notifier further states that their own research over many years indicates that spores survive for a limited time on leaves and fruits, and soon after the application a decline starts. Therefore the label recommendation is to spray three times with an interval of one week to get control of whitefly and/or thrips population. The underlying reason for this is the decline of living and infectious spores (not the increase of newly produced leaves, since whitefly larvae are present on older, matured leaves). Spores do not germinate without a host insect or another nutrient source like honeydew, and do not produce mycelium just by being on a substrate, such as a leave or fruit. Hence, production of new spores is very unlikely.

Spore survival is strongly dependent on the ambient conditions, but mainly on the RH (relative humidity). Conidiospores produced by the fungus are embedded in slimeheads which protects them from desiccation. However, commercially produced and sprayed spores do not have a slime layer around them anymore and are prone to desiccation (the slime is diluted in the water and no longer around the spore). Hall (1980) studied this and states that spores freed from their slime by e.g. splash dispersal, would rapidly die through desiccation. In commercial greenhouses it is obvious that viable spore numbers on treated plants decline in time. This is also indirectly shown by Ravensberg et al (1998), who show that spores protected in an oily formulation, survive longer and infect more thrips than unprotected spores. After nine days there is no mortality anymore on thrips when spores are applied as spore powder sprayed with water (This oil formulation was never developed into a product due to other technical challenges). To conclude it can be said that initial spore numbers applied to a crop quickly decline (to less than 50% in a week) and after about 14 days (depending on abiotic factors) spore numbers will drop very fast.

The notifier stated that they recently carried out a spore persistence test on a cucumber crop. Cucumber generates the highest level of relative humidity directly in its micro-layer at the underside of the leaves and for that reason is the most suitable crop for fungal biopesticides. Spore decline is shown to occur quickly the first 7 days after the application (down to less than 50%) and after about 20 days spore numbers have declined to less than 10% (Hora and Ravensberg, 2007).

Under normal agricultural practices (i.e. with an acceptable whitefly infestation), *V. lecanii* Ve6 (KV01) is not able to grow on plants, as demonstrated above, but also not in plants. This fungus has never been reported as a plant pathogen and cannot develop in a plant. Also, numerous efficacy/phytotoxicity tests have been done for the registration of the product Mycotal and phytotoxicity has never been found

The notifier referred to a research that was performed by Gardner et al. (1984). The research concerned the persistence of viable residues on ornamental (chrysanthemum). The paper describes the use of the Ve2 strain (Vertalec), which is not relevant for this report.

In a study of Beerling et al. (1998) persistence of *V. lecanii* KV01 (Mycotal strain) was studied in chrysanthemum and rose. Leaf imprints were used for spore viability testing and bioassays with thrips were used to detect spore infectivity. Eleven days after spraying, chrysanthemum still had a high percentage of spores (75-80%) able to infect thrips. However, on rose a drastic decrease of spore viability was detected (approx. 12% on day 8, and 1-2% on day 15).

Beerling et al. (1998) indicated that the longevity of spores on leaves depends on different factors. Firstly, longevity is probably species and strain specific. Secondly, the climatic conditions at the leaf surface level are important, and thirdly, specific chemicals produced by the plant may affect the viability of spores. This is supported by the other studies submitted by the notifier. Thus it can be concluded that in none of the studied plants (under normal practical conditions) an increase in CFUs was observed on either the leaves or fruits, and in many plants a decrease in CFUs of 50% or more was observed 1 week after spraying. This indicates that the level of residues on plants will never exceed the initial spraying level.

Conclusion

With regard to the persistence and multiplication of viable residue on (edible parts of) crops, it can be concluded that an increase of spore numbers or mycelium is deemed not to occur under practical conditions and spore numbers decrease quickly over time, on both leaves and fruits.

Exposure to consumers

The notifier refers to the very limited study of Quinlan already described in B.2.1.5. In this study tomato fruits had been dipped in a spore suspension of 2.5 g/L Mycotal and stored at 22°C for 13 days. It was reported that no fungal growth was visible on the tomatoes (Quinlan, date unknown, source unknown,19XX a). Since spores are not visible by eye, the RMS considered this information of no use to prove that no viable residues are present on edible part of crops.

In the study of MacQueen & Quinlan described above, the number of colonies on tomato fruit were about 40% of the original count up to 12 days after application (MacQueen & Quinlan, date unknown, source unknown 19XX a). Number of colonies on leaves was a 30-fold increased on day 12. The study is of a very poor quality, making it impossible to assess whether these results give a representative picture of the multiplication of spores after application. The notifier indicated that this study is not very representative for a commercial application, since under practical conditions the level of whitefly infestation in this study (i.e. more than 300 whiteflies per plant and sooty mold damage was becoming serious) is unacceptable for a grower. The high level of whitefly caused a high amount of honeydew, which in turn was used by *Verticillium lecanii* as a nutrient to grow on; this explains the increase of *V. lecanii* CFUs after 10-12 days on the leaves.

Based on the current information available, it cannot be excluded that exposure of humans to viable residues (both spores and mycelium) may occur. However, the level of residues on plants will never exceed the initial spraying level, hence will not to exceed the levels tested in the toxicity studies. Therefore, the residual spores by itself are considered safe to consumer as in the animal studies no toxicity effect was observed (on testing the spores).

New data 2016

No new data is to be submitted under this point.

Previously submitted information with additional explanation with respect to the Skrobek et al 2005 study is considered to be acceptable to cover current requirements.

B.7.3 Summary and evaluation of residue behaviour

Information from the original DAR

With regard to the persistence and stability of metabolites on (edible parts of) crops, it can be concluded that the destruxins itself, when applied as such, were still present on tomato leaves after two weeks under greenhouse conditions. However, no toxins are expected to occur during and after application of Mycotal. Skrobek et al. (2005 submitted) discovered that some extracts from Mycotal, unformulated

spores and tomatoes and cucumber after foliar treatment contained unidentified compounds that were cytotoxic in the insect Sf-9 cell line. Destruxins could not be detected in the extracts. The amounts found are very low, and since the mode of action of *V. lecanii* is not considered to be based on toxins, the relevance of these unidentified compounds is questioned. It was concluded that investigations into the occurrence of any possible metabolite produced by *V. lecanii* Ve6 should be considered irrelevant for the risk assessment.

With regard to the persistence and multiplication of viable residue on (edible parts of) crops, it can be concluded that an increase of spore numbers or mycelium on leaves and fruits is deemed not to occur under practical conditions and spore numbers decrease quickly over time. As in the animal studies no spore-related toxicity was noted and the residual levels are expected not to exceed the levels tested, no additional studies are required.

It can therefore be concluded that no risk for the consumer due to the exposure to *Verticillium lecanii* Ve6 is currently expected.

New data 2016

No new data is to be submitted under this point.

Previously submitted/not published information with additional explanation with respect to the Skrobek et al 2005 study is considered to be acceptable to cover current requirements.

B.7.4 References relied on

New data 2016

A literature review on residues of *Lecanicillium muscarium* Ve6 (19-79) in or on treated products, food and feed was conducted to identify recent (from 2006 onwards) literature which may affect the assessment on human health, animal health and/or the environment, with the special consideration of residues in or on treated products (Scholze 2016). The report summarises an evaluation of a literature search performed according to the EFSA document. The search strategy was based on relevance criteria and reliability criteria. 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. The search terms used were *Lecanicillium* or Mycotal, or *Verticillium lecanii*, food OR feed OR consumer OR residue?,,? OR ?efficacy? OR ?efficiency? and 2006-2016. Obtained references were first subjected to a *rapid assessment* based on title and the abstract. Summary records that appeared to be relevant passed to a second step in which a detailed assessment of full text documents was conducted. Only studies that were considered relevant were assessed for

reliability (see relevance and reliability criteria). In total 156 references were retreived after all searches of peer-reviewed literature. After the rapid assessment, four references were identified as being potentially relevant to subjected to a *detailed assessment* of the full-text documents. 152 references were excluded basing on their title and abstracts. Basing on the full text, 3 articles were considered as relevant for Section 6; Vidal 2015, Anderson 2007 and Gurulingappa 2010. The non relevant article was about the viability of *Lecanicillium muscarium* on sorghum, rice, wheat and maize in natural conditions (and not as culture media). Furthermore, the taxonomy of the used strain was not clear.

Relevance criteria:

- Property investigated was relevant for data requirements of Regulation (EC) No 1107/2009
- Subject relevant for residues of *Lecanicillium muscarium* analysed on products, food and feed
- Subject relevant for residues of *Lecanicillium muscarium* occurrence on plants
- Test species/system relevant to the residues on products, food and feed
- Application on crops and consumer risk
- Relevant crop / trial location

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; appropriate statistical methods used
- Conclusions robust and clear
- Definition of endpoints
- Presentation of result
- Guideline compliance

Comment RMS: The RMS questions whether the article about the viability of *Lecanicillium muscarium* on sorghum, rice, wheat and maize in natural conditions (and not as culture media) is not relevant. The article could give more information on the fate of *Lecanicillium muscarium* (independent on the strain) in the environment. Therefore, RMS requires more explanation from the applicant.

Applicant submitted more explanation: Amaker et al. (2009): the article focusses on getting a substrate suitable for mass production of Verticillium muscanii for later use as biocontrol agent. The fungus was grown on different carriers in culture broth in the lab at 27°C. Growth was not tested on the substrates alone but always in combination with culture broth. It was therefore concluded by Scholze (2016) that

the information provided is not relevant with regard to persistence/growth in natural environments and treated plants. The reference can be submitted upon request. RMS accepted the explanation.

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed* Y/N	Owner **
Annex II Data and Information					
IIM 6.1/01	Scholze, I.	2016	LITERATURE REVIEW ON LECANICILLIUM MUSCARIUM VE6 (19-79): RESIDUES IN OR ON TREATED PRODUCTS, FOOD AND FEED Koppert, 2191392-MA-06-01 GAB Consulting GmbH, Heidelberg, Germany GLP/GEP: no Published: no	Y New data for active ingredient, not previously submitted nor evaluated	KBS
IIM 6.1/02 B.7.1	Groeneveld, C.N.	2001a	Residue data *Verticillium lecanii*. OpdenKamp Adviesgroup, Koninginnegracht 23, 2514 AB, The Hague, The Netherlands. Koppert Beheer BV KO\DEN01.1703/1, pp. 9-10 - unpublished statement	Y	KBS
IIM 6.1/03 B.7.1	Vidal, S., Jaber, L.R.	2015	ENTOMOPATHOGENIC FUNGI AS ENDOPHYTES: PLANT-ENDOPHYTE-HERBIVORE INTERACTIONS AND PROSPECTS FOR USE IN BIOLOGICAL CONTROL -, not applicable Current Science, 109, 46-54 GLP/GEP: no Published: yes	N	-
IIM 6.1/04 B.7.1	Anderson, C.M.T., McGee, P.A., Nehl, D.B., Mensah, R.K.	2007	THE FUNGUS LECANICILLIUM LECANII COLONISES THE PLANT GOSSYPIUM HIRSUTUM AND THE APHID APHIS GOSSYPII -, not applicable Australasian Mycologist, 26, 65-70 GLP/GEP: no Published: yes	N	-

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IIM 6.1/05 B.6.1	Gurulingappa, P., Sword, G.A., Murdoch, G., McGee, P.A.	2010	COLONIZATION OF CROP PLANTS BY FUNGAL ENTOMOPATHOGENS AND THEIR EFFECTS ON TWO INSECT PESTS WHEN IN PLANTA -, not applicable Biological Control, 55, 34-41 GLP/GEP: no Published: yes	N	-
IIM 6.1/06 B.7.2.1	Butt, T.M., Skrobek, A., Wang, C., Shah, F.A., Ben El Hadj, N.	2004	RAFBCA Partner 01, University of Wales, Swansea, UK, Final Report 01.11.01-31.10.04. University of Wales, Swansea, UK, Final Report 01.11.01-31.10.04.; School of Biological Sciences, University of Wales Swansea, Singleton Park, SA2 8PP, UK. - QLK1-2001-01391 - unpublished statement	Y	KBS
IIM 6.1/07; B.7.2.1 IIM 6.5; B.7.3	Skrobek, A., Ravensberg, W.J., Ben El Hadj, N., Vey, A., Butt, T.M.	2005	Studies on the production of destruxins by the biological insecticides Mycotal® and Vertalec®. School of Biological Sciences, University of Wales Swansea, Singleton Park, SA2 8PP, UK, Koppert Biological Systems, POB 155, 2650 AD Berkel en Rodenrijs, The Netherlands, Institut National de la Recherche Agronomique (I.N.R.A.), Unité de Recherche de Pathologie Comparée, 30380 St Christol les Alès, France. Koppert: not applicable Koppert Biological Systems GLP/GEP: no Published: no	N	KBS
IIM 6.1/08 B.7.2.2	M.D. MacQueen, R.J. Quinlan	19XX	Residual *Verticillium lecanii* on the leaves and fruit of a tomato crop treated with Mycotal microbial Insecticide. University of Reading, Reading, Berks, UK Koppert Beheer BV - Not GLP unpublished report	Y	KBS
IIM 6.1/09 B.7.2.2	Koike, M. et al.,	2004	*Verticillium lecanii* (*Lecanicillium* spp) as epiphyte and its application to biological control of arthropod pests and diseases. IOBC/WPRS Bulletin 27(8): 41-44.	N	-

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IIM 6.1/10 B.7.2.2	Hall, R. A.	1980	Effect of relative humidity on survival of washed and unwashed conidiospores of *Verticillium lecanii*. Ecol. Applic. (1)3: 265-274.	N	-
IIM 6.1/12 B.7.2.2	Ravensberg, W.J.,R. van der Pas and E. Cryer,	1998	Insect pathogenic fungi for environmentally-friendly pest control in the glasshouse; investigating oil formulations. !OBC/WPRS Bulletin 21(4): 129-132.	N	-
IIM 6.1/13 B.7.2.2	Hora, K. and W. Ravensberg	2007	Persistence of Mycotal spores under greenhouse conditions on cucumber. Internal report Koppert BV.	Y	KBS
IIM 6.1/14 B.7.2.2	Gardner, W.A., Oetting, R.D., Storey, G.K.	1984	Scheduling of *Verticillium lecanii* and benomyl applications to maintain aphid (Homoptera: Aphidae) control on chrysanthemums in greenhouses. University of Georgia, College of Agriculture Experiment Stations, Georgia Station, Griffin, Georgia 30212, USA. - Journal of Economic Entomology 77, pp. 514-518 - Published report	N	KBS
IIM 6.1/15 B.7.2.2	Beerling, E.A.M., Joosten, N.N., Tolsma, J., Fransen, J.J.	1998	Studies of entomopathogenic fungi for control of thrips and three aphid species on glasshouse ornamentals, final report of participant 04. Research Station for Floriculture and Glasshouse Vegetables (PBG), Linnaeuslaan 2a, 1431 JV Aalsmeer, the Netherlands Koppert Beheer B.V. AIR3 CT94-1352 Not GLP unpublished report	N	KBS
IIM 6.1/16 B.7.2.2	Quintana, R.J.	19XX	Effect of MYCOTAL on fruit ripening and storage. University of Reading, Reading, Berks, UK. Koppert Beheer B.V. - Not GLP Unpublished	Y	KBS

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed* Y/N	Owner **
	Butt, T.M., Hadj, N.B.E., Skrobek, A., Ravensberg, W.J., Wang, C., Lange, C.M., Vey, A., Shah, U.K. and Dudley, E.,	2009	Mass spectrometry as a tool for the selective profiling of destruxins; their first identification in Lecanicillium longisporum. Rapid Communications in Mass Spectrometry, 23(10), pp.1426-1434.	N	-
	Dudley, E., Wang, C., Skrobek, A., Newton, R.P. and Butt, T.M.,	2004	Mass spectrometric studies on the intrinsic stability of destruxin E from Metarhizium anisopliae. Rapid communications in mass spectrometry, 18(21), pp.2577-2586.	N	-
	Kouvelis, V.N., Wang, C., Skrobek, A., Pappas, K.M., Typas, M.A. and Butt, T.M.,	2011	Assessing the cytotoxic and mutagenic effects of secondary metabolites produced by several fungal biological control agents with the Ames assay and the VITOTOX® test. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 722(1), pp.1-6.	N	-
	Skrobek, A. and Butt, T.M.	2005	Toxicity testing of destruxins and crude extracts from the insect-pathogenic fungus Metarhizium anisopliae. FEMS microbiology letters, 251(1), pp.23-28.	N	-
	Skrobek, A., Boss, D., Défago, G., Butt, T.M. and Maurhofer, M.	2006	Evaluation of different biological test systems to assess the toxicity of metabolites from fungal biocontrol agents. Toxicology letters, 161(1), pp.43-52.	N	-
	Skrobek, A., Shah, F.A. and Butt, T.M.	2008	Destruxin production by the entomogenous fungus Metarhizium anisopliae in insects and factors influencing their degradation. BioControl, 53(2), pp.361-373.	N	-

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

***: Owners' code identifications and names (Code identification: KBS Name: Koppert Beheer Systems).*