ANNEX B

Paecilomyces fumosoroseus Strain Apopka 97

B.1 Identity

B.1.1 Identity of the organism (Annex IIB 1)

B.1.1.1 Applicant (name, address, etc.) (Annex IIB 1.1)

Thermo Trilogy Corporation
7500 Grace Drive
Columbia, Maryland 21044
United States of America

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B.1.1.2 Manufacturer (name, address, including location of plant) (Annex IIB 1.2)

Thermo Trilogy Corporation
7500 Grace Drive
Columbia, Maryland 21044
United States of America

B.1.1.3 Name and species description (Annex IIB 1.3)

B.1.1.3.1 Scientific name and taxonomic grouping (Annex IIB 1.3.1)

Genus, species, related species :
The genus *Paecilomyces* is closely related to *Penicillium*. The characteristics used to separate these two genera are that species of *Paecilomyces* lack green coloured colonies and have short cylindrical phialides which taper into long necks. The latest revision of this genus was by Samson (1974) in which he placed 31 species into two sections: *Paecilomyces* and *Isarioidea*. *P. fumosoroseus* belongs to the section *Isarioidea* as do several of the entomopathogenic species (Osborne and Landa, 1992).
Fourteen species in the fungal genus *Paecilomyces* are known pathogens of various arthropod and nemathode hosts found on plants and in the soil throughout the world (Samson, 1974). For each species, many strains appear morphologically similar but differ genetically and pathologically.

Specific strain:
This particular isolate of *Paecilomyces fumosoroseus* (Wize) Brown & Smith, Apopka strain 97 (PFR 97 or CG 170) was originally isolated from *Phenococcus solani* Ferris (mealy bug), on gynura in a greenhouse located in Apopka, Florida, by Dr. Lance Osborne, Agriculture Research Center, University of Florida, Apopka, Florida, in 1986 . A pure culture of this strain, called at that time UK-1, was sent for identification to the USDA Agriculture Research Service at the Boyce Thompson Institute under the accession number ARSEF 2247, and to Dr. R.A. Samson of the Centraalbureau voor Schimmelcultuur in the Netherlands. (Dr. Samson monographed the taxonomy of *Paecilomyces fumosoroseus*)
The culture has been identified as a clean, pure isolate of *Paecilomyces fumosoroseus*. In February 1988, a pure culture of this strain was deposited at the American Type Culture Collection (ATCC) under the name *Paecilomyces fumosoroseus* Apopka ATCC 20874. (Humber, 1996a; Humber 1996b; Samson, 1987; USDA Agricultural Research Service, 1992)

Table B.1.1.3.1-1 describes the growth characteristics of *Paecilomyces fumosoroseus* Apopka strain 97 compared to *P. lilacinus* and *P. variotii* and compares the fruiting bodies and structural characteristics of the different *Paecilomyces* strains. *Paecilomyces fumosoroseus* Apopka strain 97 can be easily distinguished from *P. lilacinus* and *P. variotii* which are known to be pathogenic to vertebrates and to humans.

Table B.1.1.3.1-1 : Taxonomic comparison of characteristics of several *Paecilomyces*.

Species	***Paecilomyces fumosoroseus***	***Paecilomyces lilacinus***	***Paecilomyces variotii***
Growth characteristics :			
Colony color on plate	dull pink	purple to pale greyish violet	olive buff to light yellow olive
Optimal growth temp. °C	25-28	35	35-40
Growth at 35°C	No	Yes	Yes
Conidia :			
Colour	Pink	Purple	Yellow Brown
Size	3-4 x 1-2 µm	2.5-3 x 2-2.2 µm	3.2-5 x 2-4 µm
Shape	cylindrical with oval or rounded end	elliptical	elliptical
Chlamydospores	No	No	Yes
Conidiophores :			
Structure	simple venticle or solitary phialides	groups of brush like whorls (penicilli)	multiple brush like whorls (penicilli)
Number of phialides per branch	4 to 6	2 to 4	2 to 7
Branches	random on short limbs	opposing of uniform length	random, irregular on long limbs
Phialides :			
Size	1-2 µm base 0.5 µm neck	2.5-3 µm base 1 µm neck	3.2-5 µm base 2-4 µm neck
Shape	globose or ellipsoidal base tapering slowly to a long distinct neck	long basal port tapering slowly to long neck	cylindrical or ellipsoidal base tapering abruptly to long cylindrical neck

PFR-MUP product chemistry (Eyal, 1994a)

B.1.1.3.2 Definition of the micro-organism (in case of taxonomical difficulties) (Annex IIB 1.3.2)

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B.1.1.3.3 Appropriate test procedures and criteria used for identification (e.g. morphology, biochemistry, serology) (Annex IIB 1.3.3)

Morphology of P. fumosoroseus:

When *P.fumosoroseus* is grown on agar media or in insects, the following observations have been made.
Colonies on malt-agar growing moderately fast, attaining a diameter of about 4 cm within 14 days at 25° C, consisting of a basal felt with raised floccose overgrowth, powdery when freshly isolated; in some isolates pink synnemata occur which may be branched; white at first, remaining so or changing to pink shades, especially when sporulating abundantly. Reverse uncoloured or yellow. Odour absent. Vegetative hyphae smooth-walled, hyaline, 1.5-3.5 µm wide. Conidial structures mostly complex, consisting of erect conidiophores arising from submerged or laterally from aerial hyphae. *Conidiophores* mono- or synnematous, up to 100 µm in length, 1.5-2 µm wide, smooth-walled, hyaline, consisting of verticillate branches bearing whorls of 4 to 6 phialides.
Phialides 5.7-8 x 1-2 µm, with a globose or ellipsoidal basal portion which tapers into a long distinct neck, about 0-5 µm wide.
Conidia cylindrical to fusiform, smooth-walled, hyaline to slightly pink, 3-4 x 1-2 µm. Chlamydospores absent.

On insects the fungus produces mononematous conidiophores or distinct but loose synnemata.
Synnemata erect, branched, appearing pink and powdery due to the conidia, up to 3 cm in length and 400 µm in diameter. Conidiogenous elements inflated. Conidiophores 2.5-4 µm wide; verticillate branches more or less globose or broadly cylindrical, 4-9 x 3-4.5 µm. Phialides with a globose basal portion, 2.3-3.5 µm wide.
The morphological characteristics used for classification of *Paecilomyces* species do not unambiguously resolve new isolates into clearly defined species and provide no isolate-level characterization. (Samson, 1974)

Paecilomyces fumosoroseus can exist in several morphological stages as follows:

Morphological stage	Culturing media
Conidiophores - Conidia	Solid media
Blastospores	Submerged culture
Mycelium	Solid and submerged culture

- *Isozyme characterization* (Tigano-Milani et al., 1995a)*:*
Ten monosporal fungal isolates of *Paecilomyces fumosoroseus* (Wize) Brown and Smith and 14 isolates of *Paecilomyces lilacinus* were characterized biochemically by separating mycelial proteins using isoelectric focusing(IEF).
Eleven isoenzyme systems were used to detect polymorphism among these isolates. The enzyme profiles for the enzyme systems Acid phosphatase, Phosphogluconate dehydrogenase and Phosphoglucomutase showed no common banding position for isolates of *P.fumosoroseus* and *P.lilacinus*. Isolates of *P.fumosoroseus* were separated into two phenetic groups with approximately 40% similarity.The strains of *Paecilomyces fumosoroseus* CG123, CG170, CG203, CG204, CG205, CG325 and CG344 (isozyme cluster 1) were identical for the characters analyzed, as were the strains of *Paecilomyces fumosoroseus* CG297, CG323 and CG326 (cluster 2). These groups did not correlate by geographical origin or host, and the low similarity between them indicates the possibility of subgroups within the species, or even the existence of an aggregate species.
The most highly virulent isolates of *P.fumosoroseus* (CG170, CG204, CG344) and *P.lilacinus* (CG177, CG303, CG332, CG333) against rootworm and (or) rootknot nematode eggs were not related to any specific cluster obtained by isozymes analysis.
The 14 *P.lilacinus* isolates, collected from different geographic locations and from insects, clustered together in a homogeneous group at more than 85% similarity. Both taxa exhibited approximatively 30% similarity.
Conclusion:
These results support the taxonomic separation of *P.fumosoroseus* from *Paecilomyces lilacinus* based on morphological differences and suggest that 11 isoenzymes are useful to differentiate between typical *P.fumosoroseus* and *P.lilacinus* strains. There was no correlation between isozymes and pathogenicity of the different strains.

- *Genetic markers :*
Arbitrarily primed PCR (Polymerase Chain Reaction) and PCR using tRNA consensus primers were used to evaluate the genetic variability among 27 *P. fumosoroseus* isolates, 15 of which came from the same host, *Bemisia tabaci*, 1 from *P.lilacinus* isolate, 9 previously unidentified *Paecilomyces* isolates. Fifteen 10-mer oligonucleotide primers of arbitrary sequence revealed 322 scorable binary characters.

Phenetic and phylogenetic analyses of 322 arbitrarily primed PCR characters and 107 tRNA-PCR characters revealed polymorphism within isolates morphologically classified as *P. fumosoroseus*.
Principal coordinates and cluster analysis showed that most of the *P. fumosoroseus* and *Paecilomyces sp.* isolates were in three phenetic groups with >65% internal similarity from phenetic analysis of arbitrarily primed PCR characters. Two of the three phenetic groups were easily distinguished by a phylogenetic analysis of tRNA-PCR characters and closely related (76% similarity) with the third group quite different (only 14% similarity) from the first two. This level of divergence suggests that what is classified as *P. fumosoroseus* likely represents a species aggregate.

Large genetic variation (over time and within a same epizootic population) of the isolates infecting whiteflies was observed.
Isolates from *Bemisia tabaci* (from India and Pakistan) were represented in two of the three groups, and different genotypes were identified even when they were isolated from the same epizootic population collected in one field. These results clearly demonstrate that more than one genotype was found parasitizing whiteflies at the same time.There was no evidence of host-specific selection of genotypes, as has been shown in other entomopathogenic fungi.
Isolates infecting *Bemisia tabaci* (from Florida) collected in the same area but in different years revealed genetic variability.

The phenetic groups did not correlate with geographical origin or host species.
Group 1 isolates had different geographical origins and were isolated from diverse substrates including leaves, soil, and insects (see table B.5.1.1.3.3-1).

Table B.1.1.3.3-1: Origin of isolates of phenetic group 1.

Isolate	**Host or substrate**	**Location**	**Year**
P.fumosoroseus			
CG35 (ARSEF3480)	*Cyperus rotundus*	Brasilia/DF/Brazil	1991
CG170 (PFR97)	*Pseudococcus sp.*	Apopka/FL/USA	1989
CG202 (ARSEF1867)	*Musca domestica*	Brittany/France	1985
CG205 (5748)	*Bemisia tabaci*	Gainesville/FL/USA	1992
CG259	*Eurhizococcus brasiliensis*	Caxias Sul/RS/Brazil	1992
CG296 (ARSEF 1532)	*Adelphocoris sp.*	Orvieto/Italy	1984
CG298 (ARSEF 1577)	*Hyphantria cunea*	Cade/italy	1984

Isolate CG170 (PFR97) belongs to the phenetic group 1. However, despite their diverse origins, isolates of group 1 were clustered with a very high similarity (>88%) and therefore represent a highly homogeneous group. The average pairwise similarity within this group was 97%.
Phenetic groups 1 and 2 were closely related and a phylogenetic hypothesis generated using products amplified by tRNA consensus primers showed that these groups were monophyletic (Tigano-Milani et al., 1995b).

Electophoretic karyotypes of three isolates of *P. fumosoroseus* showed that the number (6) and size of their chromosomes were similar, but they did not bear the same genetic information (Shimizu et al., 1993).

B.1.1.3.4 Common name or alternative and superseded names (Annex IIB 1.3.4)

Prior to the taxonomic revision of Samson R. (1974) *Paecilomyces fumosoroseus* (Wize) Brown & Smith may be identical with the following fungi strains :

Isaria fumosorosea Wize - Bull. Int. Acad. pol. Sci. Lett. (Classe Sci. math. nat.) 1904.

Spicaria fumosorosea (Wize) Vassiljevski - Morbi Plant. p. 146. 1921 = *Paecilomyces fumosoroseus* (Wize) Brown Smith - Trans. Br. mycol. Soc. 40 : 67. 1957.

Spicaria aphodii (Vuill.) - Bull. Séanc. Soc. Sci. Nancy, Sér. 3, 11 : 24. 1910 = *Penicillium (Spicaria) aphodii* (Vuill.) Biourge - La Cellule 33: 100. 1923.

Spicaria cossus Portier & Sartory - C. r. Séanc. Soc. Biol. 79 : 700. 1916. = *Paecilomyces cossus* (Portier & Sartory) Brown & Smith - Trans. Br. mycol. Soc. 40 : 74. 1957.

Paecilomyces hibernicus Kennelly & Grimes - Scient. Proc. R. Dubl. Soc. 19 : 513. 1930.

Paecilomyces isarioides Inagaki - Trans. mycol. Soc. Japan 4 : 2. 1962.

B.1.1.4 Composition of the material used for manufacturing of formulated products (Annex IIB 1.4)

B.1.1.4.1 Microbiological purity of the micro-organism (Annex IIB 1.4.1)

In order for a sample to pass quality control specifications, it must contain at least 1. 10^8 CFU/ml in the fermentation broth at the end of the fermentation.

Table B.1.1.4.1-1 : Final fermentation broth - PFR-MUP - 'technical' Paecilomyces

Components	Nominal %
Paecilomyces fumosoroseus Apopka Strain 97 Blastospores and Mycelia	7.0 %
Inert ingredients and water	93.0 %
TOTAL	100.0 %

B.1.1.4.2 Identity and content of impurities, additives, extraneous micro-organisms (Annex IIB 1.4.2)

Extraneous micro-organisms:
There are two stages at which the contamination load is monitored : after fermentation and after formulation.
No contaminates are allowed during the fermentation process. The fermentation process is monitored from inoculation to harvest by microscopic analysis and plating. If any form of microbial contaminate is detected the fermentation is sterilized and destroyed. Similarly, the formulation process is monitored for microbial contamination.The range of contamination is at 10^1 to 10^2 CFU/g as was reported from the 12 PFR-MUP production batches which passed QC during 1995. This low contamination level is due to the production method based on the direct granulation of the sterile PFR-MUP biomass from the fermentor onto pasteurized/sterile food ingredient formulation carrier.
The low levels of microorganisms which are detected on the agar plate are subject to fatty acid analysis by an outside lab to identify the nature of the contaminant. Fatty acid analysis conducted on batches that have been produced in 1995 revealed that only *Bacillus subtilis* is present at levels of 10^2 CFU/g in the production batches. According to the Bergey's Manual this bacteria species is recognized as a nonmammalian pathogen.

The method of analysis will reject:
1. Batch of PFR-MUP containing above 10^3 CFU/g of contaminants or,
2. Batch of PFR-MUP containing bacteria which are known to be pathogenic, e.g.,
   Salmonella/Shigella spp > 100 CFU/g
   Coliform > 100 CFU/g,
   Pseudomonas aeruginosa > 100 CFU/g (Eyal, 1994c)

Secondary metabolites:
During the growth of *Paecilomyces fumosoroseus* Apopka strain 97 in submerged culture, several common metabolites would be expected to form. These metabolites include lactic acid, acetic acid, carbon dioxide, ethanol, and various proteins, protein peptides, nucleic acids, and polysaccharides. Such metabolites are generally and routinely formed when fungi are cultured in submerged media. None of theses metabolites is considered to be of any toxicological significance.

The strain was screened by the notifier for secondary metabolites produced by conidia as well as by mycelia of the fungus in still culture and submerged culture.The main interest was to determine if this fungus strain produces aflatoxins, beauvericins or new metabolites.

- Production of aflatoxins:
Mycelia and conidia were used for the analysis. The strain was cultured for 21 days as a still culture on the surface of liquid Sabouraud media. The mycelial cake obtained after 21 days was extracted with methanol/water solution and dried.
The extract was analyzed by HPLC and with a kit for the detection of aflatoxin. Results from the HPLC analysis as

well as from the aflatoxins kits revealed that no aflatoxins were detected at the ppb level.

- Production of beauvericin:
The strain of *Paecilomyces fumosoroseus* was grown for 3 days at 27°C in submerged culture in a medium containing peptone (1%), yeast extract (0.2%), dextrose (5%) in water. The fungal mycelium (1.5 kg) was extracted with methylcetone. Combined extracts were dried.The residue was extracted with $CHCl_3$. After TLC separation, a single crystalline compound was obtained which was identified as beauvericine on the basis of MS, NMR and rotary power measurements (Bernardini et al.,1975).
No beauvericin was detected in the mycelium extract of *Paecilomyces fumosoroseus* Apopka strain 97, however the comparison revealed the presence of beauverolides.

- Production of beauverolides :

Data from open literature show that beauverolides were isolated from *P. fumosoroseus* (Wize) Brown & Smith (PFR97-Apopka, ATCC 20874) after stationary culture for 21 days at 26°C in 500 ml conical flasks containing Sabouraud medium. A 72 h preculture was used as inoculum. Isolation of beauverolides was perfomed from mechanically separated and roughly fragmented mycelium (200 g), which was extracted twice with methanol overnight.The volume of the combined extracts were reduced to 400 ml in vacuo, H_2O was added with stirring and the suspension formed was allowed to stand overnight at 5°C. The crude mixture of depsipeptides was separated by centrifugation and dried in vacuo. TLC was used to monitor the presence of compounds of a peptidic nature. Final purification was carried out by LC .
Dilution of methanolic extracts of the mycelium of *P.fumosoroseus* with water afforded a white microcristalline coagulate. GC-mass spectral analysis of the hydrolysated coagulate revealed the presence of branched 4-methyl-3-hydroxy acids and some amino acids in all samples. Direct probe EI-mass spectra indicated that the sample was a mixture of various beauverolides (ca 1% of the dry weight of mycelia). Two main constituents of the mixture were obtained by preparative reversed-phase liquid chromatography and were designated as beauverolides L and La. Beauverolide L accounts for 60% of the total amount of the produced beauverolides. The second, minor component of the material, beauverolide La, coeluted partly with beauverolide L on the RP-HPLC column. The structures of these cyclic tetradepsipeptides, cyclo-[3-hydroxy-4-methyldecanoyl-L-phenylalanyl-L-alanyl-D-leucyl), and cyclo-(3-hydroxy-4-methyldecanoyl-L-phenylalanyl-L-alanyl-Dallo-isoleucyl) were deduced from HPLC and GC-mass spectrometric analyses (Jegorov et al, 1994).

- Beauverolides L and La are water insoluble which make difficult to dose them under laboratory conditions and reduces the potential contact between metabolite and host to minimum.
- No antibacterial nor antifungal activities have been determined.
- No direct toxic effect of beauverolides L and La was determined either in insects or in other invertebrates injected or fed by metabolites.
- The injection of particles coated by metabolites induced in treated *Galleria mellonella* larvae some induction of humoral immunity (see below). (Matha, 1996)

Beauverolide L was first isolated by Jegorov et al., (1990) from the mycelium of the entomopathogenic fungus *Beauveria brongniartii.*
In spite of high abundance in fungal extracts no data about beauverolides activity are available. Several fungal metabolites are known to induce strong immunomodulatory effects in vertebrates which are in fact not accompanied by any direct toxicity. The influence of the intrahaemocoelic injection of both beauverolides L suspension and beauverolide L-coated particles on *Galleria mellonella* immune responses were investigated. beauverolide L was isolated by methanolic extraction from a mycelium of *Paecilomyces fumosoroseus* Apopka strain 97. Structure of beauverolide L was confirmed by MS and 2D-^{1}H and ^{13}C-NMR spectroscopy. Larvae of *Galleria mellonella* were injected with a suspension of beauverolide L (10-30 µg of particles per larva). Intrahaemocoelic injection of any tested concentration or formulation of beauverolide L did not induce direct toxic effect but even a single dose activated both cellular and humoral immunity of treated larvae.Cellular immune reactions of larvae injected with beauverolide L-coated particles were characterized by increased phagocytosis and rapid attachment and spreading of both granular cells and plasmocytes followed by the formation of nodules with partial melanization. This effect of

beauverolide L could be considered as specific one due to the fact that practically no response was determined in oil injected larvae. The single dose of beauverolide L was followed by the synthesis of antibacterial proteins in the haemolymph of treated larvae. Soluble metabolite revealed a dose dependent induction of both anti-E coli and lysozyme activity.
These results showed strong stimulatory effect of beauverolide L on the immune system of *G.mellonella* larvae (Matha et al, not accepted for publication : the reviewers did not recognise the effect specific and significant enough).
Supraphysiological concentrations of beauverolides L and La did not reveal any direct toxic or other indirect biological effects and could be evaluated as non-toxic compounds.

The use of *Galleria mellonella* as indicator of immunotoxicity is a classical test which demonstrates toxic properties of secondary metabolites. Destruxin E, which has a chemical structure comparable to beauverolides, is immunotoxic in this test. This cyclodepsipeptidic mycotoxin , is composed of five amino acids and an α-hydroxy acid and exhibits a wide range of biological properties , such as cytostatic or cytotoxic effects on mouse leukemia cells, immunodepressive activity toward insect models and insecticidal effects. Moreover, in *Galleria mellonella* larvae, a tetanic paralysis triggered by destruxin ingestion was demonstrated (Cherton et al., 1991).

Rationale of the notifier concerning the conclusion that Beauverolides are absent in the active substance (PFR-MUP) or in the end product of PREFERAL WG: (Anonymous, -)

- The active substance (PFR-MUP) is composed of blastospores. Blastospores are spores that bud from the mycelium. The mycelium is formed a short time after fermentation process has started (during the exponential phase). However, after 72 h the blastospores start to bud from the mycelium and the concentration of blastospores increases substantially. At the end of the fermentation, after consumption of all nutrients, the fermentation broth contains mainly (99%) blastospores (between 1-3 x 1^{9} blastospores per liter) and traces of mycelium (less than 1%). Beauverolides have only been detected during the cultivation of *Paecilomyces fumosoroseus* Apopka strain 97 in still culture but not in liquid fermentation.

- The absence of beauverolides in the fermentation broth (PFR-MUP) or in the end use product was determined by the notifier by using a HPLC analysis. (Hull, 1992)
Samples for HPLC analysis were prepared according to the protocol described by Jegorov et al.(1994).
Briefly, 250 g of the WG formulation (batch 119) were extracted by 1 l of methanol overnight. The extract was concentrated in vacuo and washed with 400 ml distilled water. Suspension formed was allowed to stay overnight at 5°C, the solid fraction was removed by centrifugation and dried in vacuo. Crude extract corresponding to 1 g formulation was dissolved in methanol and separated by using HPLC.
The mixture of available natural beauverolides consisting approximatively of 30% beauverolide L, 30% beauverolide La and of 40% of others beauverolides, specially phenylalanin containing beauverolides was used to calibrate HPLC column.
Results of the analysis of crude extract of the WG formulation, showed the presence of some minor peaks, however they exclude the presence of any of known beauverolides or even any compound having the same retention time as standard mixture of beauverolides

- Secondary metabolites are frequently produced during special growth phase or under special growth conditions. Difference between still culture and liquid fermentation:
During still culture, a rich medium composed of Sabouraud medium (rich in peptones and amino acids) is used and the still culture takes place for a long period of time- 21 days. The fermentation uses completely different media and it is much shorter (5 days maximum). Still culture conditions cannot be reproduced in a stirred fermentation as "dead space". If such dead spaces exist in the fermentor, such conditions only occur for a very short time (maximum length of the *Paecilomyces fumosoroseus* fermentation is 5 days) and the medium in which these dead spaces occur is completely different from the medium used in still culture.

- Another important factor why no beauverolides are produced in the fermentation is the high biomass level. It is known in industrial fermentation that for production of primary of secondary metabolites, overproduction of biomass reduces product yield. Overproduction of biomass delays the onset of secondary metabolite production. In the case of PFR fermentation, the fermentation conditions are optimized in order to maximize the biomass (blastospores),

which is the active substance. In still culture the conditions are different and due to the long cultivation time as well as the low biomass, secondary metabolites are more likely to be formed.

B.1.1.4.3 Analytical profile of batches (Annex IIB 1.4.3)

Confidential information. See Annex C.

B.1.2 Identity of the preparation PREFERAL WG (Annex IIIB 1)

B.1.2.1 Applicant (name, address, etc.) (Annex IIIB 1.1)

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B.1.2.2 Manufacturer of the preparation and the micro-organism(s) (Annex IIIB 1.2)

Thermo Trilogy Corporation
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United States of America

B.1.2.3 Trade name or proposed trade name and manufacturer's development code number (Annex IIIB 1.3)

PREFERAL WG

PFR-97^{TM} 20% WDG

Company code number : 4775

B.1.2.4 Detailed quantitative and qualitative information on the composition of the preparation (Annex IIIB 1.4)

The formulation PREFERAL WG contains 20% mycelia and blastospores of *Paecilomyces fumosoroseus* Apopka Strain 97 at a concentration of 2.10^9 CFU/g

Information on other components of the formulation is confidential. All the ingredients of the formulation are approved for food use by the US EPA
See Annex C.

B.1.2.5 Physical state and nature of the preparation (Annex IIIB 1.5)

Water dispersible granule (WG)

B.1.2.6 Function (Annex IIIB 1.6)

Insecticide

B.1.3 Summary of the identity

Identification of the micro-organism :

Paecilomyces fumosoroseus belongs to the section Isarioidea and is an entomopathogenic species.

The particular isolate of *Paecilomyces fumosoroseus* (Wize) Brown & Smith, Apopka strain 97 (PFR 97 or CG 170) was originally isolated from *Phenococcus solani* Ferris (mealy bug), on gynura in a greenhouse located in Apopka, Florida, by Dr. Lance Osborne . A pure culture of this strain, called at that time UK-1, was sent for identification to the USDA Agriculture Research Service at the Boyce Thompson Institute under the accession number ARSEF 2247, and to Dr. R.A. Samson of the Centraalbureau voor Schimmelcultuur in the Netherlands.

The culture has been identified as a clean, pure isolate of *Paecilomyces fumosoroseus*. In February 1988, a pure culture of this strain was deposited at the American Type Culture Collection (ATCC) under the name *Paecilomyces fumosoroseus* Apopka ATCC 20874.

Paecilomyces fumosoroseus Apopka strain 97 can be easily distinguished from other *Paecilomyces* strains by growth characteristics, optimal growth temperature, conidia, conidiophores and phialides morphology. *Paecilomyces fumosoroseus* Apopka strain 97 was characterized for isoenzyme composition and genotype. *Paecilomyces fumosoroseus* Apopka strain 97 belongs to the phenetic group 1 concerning isoenzyme characterization and genotype. Despite the fact that this phenetic group 1 brings together isolates from diverse geographical or host origins , the group is highly homogeneous. Genetic variability was detected for the isolates infecting whitefly in Florida.

Presence of metabolites :

No secondary metabolites (beauverolides) such as mycotoxins were detected in the fermentation broth or in the end use product : secondary metabolites are produced during special growth phases or under special growth conditions such as still culture. The still culture conditions cannot be reproduced in a stirred fermentation.Still culturing takes place for a long period of time- 21 days. The manufacturing process uses completely different media and it is much shorter (5 days maximum).
Another important factor why no beauverolides are produced in the fermentation is the high biomass level.
The active substance (PFR-MUP) is composed of blastospores. At the end of the fermentation, after all the nutrients are consumed, the fermentation broth contains mainly blastospores (between 1-3 x 1^{9} blastospores per liter) and traces of mycelium.
The HPLC spectrum of PFR-MUP demonstrated that beauverolides are absent from the fermentation broth ; however, quantitative informations such as detection limit, amount injected, spiked samples are missing and should therefore be supplied.The use of methanol is not adapted for the measurements realized at 214 nm; 82% of elution solvent is methanol and the other 18% ?

The absence of aflatoxins and beauvericins production was demonstrated.

Manufacturing process - Quality control - Specification
The manufacturing process which is used for the production of the product consist of 1) production of active substance via fermentation process (PFR-MUP) and 2) formulation of the active substance via granulation process (PREFERAL WG) .
The active substance (PFR-MUP) is obtained by fermentation and is composed of blastospores (99%) and mycelium (1%). HPLC analysis of an extract shows that secondary metabolites such as mycotoxins were not produced in the active substance (PFR-MUP) nor in the formulation PREFERAL.
Detailed information on the fermentation process (production of blastospores) and the manufacturing of the formulation was provided. Information on the quality checks was provided (purity specification, presence of extraneous micro-organisms, viability of the fungus).
The formulation PREFERAL WG contains 20% mycelia and blastospores of *Paecilomyces fumosoroseus* Apopka Strain 97 at a concentration of 10^{9} CFU/g . All the other ingredients of the formulation are approved for food use by the US EPA.

ANNEX B

Paecilomyces fumosoroseus Strain Apopka 97

B.2 Biological properties of the organism - Technical properties of the preparation

B.2.1 Biological properties of the organism (Annex IIB 2)

B.2.1.1 History of the organism and its uses. Natural occurrence and geographical distribution (Annex IIB 2.1)

B.2.1.1.1 Historical background (Annex IIB 2.1.1)

Paecilomyces fumosoroseus Apopka Strain 97 was isolated from the mealybug *Phenococcus solani* on Gynura in a greenhouse located in Apopka, Florida, by Prof. Dr. Lance Osborne, Agriculture Research Center, Florida University in 1986. The patented strain was licenced to Thermo Trilogy Corporation (formerly W.R. GRACE & Co.-Conn, GRACE Biopesticide Division) who developed the production and formulation.

Further field development of *P. fumosoroseus* strain Apopka 97 has been done by Biobest, Belgium, for the control of whitefly in greenhouse vegetables, ornamentals and small fruits in Europe. This fungus will be used to support the biological control of whiteflies with the parasitoid *Encarsia formosa* and other natural ennemies like the mirid bug, *Macrolophus caliginosus.*

B.2.1.1.2 Origin and natural occurrence (Annex IIB 2.1.2)

Paecilomyces fumosoroseus is a naturally occurring fungus geographically widespread and a common entomopathogenic of insect pests. Like most entomopathogenic fungi *P. fumosoroseus* may be found in various soil types at very low densities. The '*Galleria* bait method' (Zimmermann, 1986) can be used to isolate the fungus.

The fungus has frequently been isolated from infected insects throughout the world (Brazil, Canada, Dominican Republic, China, Costa Rica, Czech Republic, France, Finland, Germany, Ghana, Greece, India, Indonesia, Ireland, Italy, Japan, Mexico, The Netherlands, Nepal, Pakistan, Philippines, Poland, Spain, South Africa, Sri Lanka, Switzerland, Trinidad, USA, Russia, Venezuela). (Sterk et al,. 1996)

B.2.1.2 Information on target organism (Annex IIB 2.2)

B.2.1.2.1 Description of the target organism (Annex IIB 2.2.1)

Paecilomyces fumosoroseus causes infections of all stages (eggs, immature stages and adults) of greenhouse whitefly (*Trialeurodes vaporariorum*)

B.2.1.2.2 Mode of action (Annex IIB 2.2.2)

Spores of the fungus germinate on the insect after the application. The fungus penetrates into the body of the insect where it develops, causing the death after 7-10 days.
In high relative moisture conditions, the fungus can grow outside of the insect and produce new spores.
The mode of action of *Paecilomyces fumosoroseus* is not fully understood.

In general, entomopathogenic fungi are seen to penetrate the wax, chitin and protein matrices of the cuticle without an obvious detrimental effect upon the insect other than perhaps a local discoloration. As the mycosis progresses, hyphae or hyphal bodies proliferate within the haemocoel. For such proliferation to succeed, an effective means of overcoming the cellular defences of the host has to be available. Plasmocytes, normally dispersed throughout the haemolymph, accumulate around the hyphal bodies and filaments to form pseudotissue masses or granulomata.With pathogens of low virulence, this system of cyst formation may contain the disease and the insect will survive ; more virulent pathogens overcome encystment, possibly by toxin production, and continue to develop within the haemocoel. Dense hyphal masses may form and mechanically disrupt the host's internal organs or pervasive hyphal activity may initiate tissue necrosis and loss of function. Toxin production could be involved at any stage of the mycosis.
Cyclic peptides are frequently isolated from entomopathogenic fungi ; *Beauveria bassiana* alone produces beauvericin, beauverolides and bassianolide, each of which have been asssociated with insecticidal activity in some way.

A popular and recurring theme in the literature of insect pathology is the concept of the *in vivo* toxin production by diverse species of entomopathogenic fungi. Indeed, a considerable number of low-molecular-weight secondary metabolites have been isolated from insect pathogens and many have been shown to possess marginal insecticidal activity.
A strain of *Verticilium lecanii* killed larvae of *Pyrausta nubilalis* before penetration of the hypodermis had been achieved. An infection by *Aspergillus tamarii* upon *Ceratitis capitata* was described and claimed that the fungus developed in a wound clot and killed the insect before any further colonisation had occurred. These observations suggest some form of toxin involvement, but observations of this kind are extremely rare. (Gillespie and Claydon, 1989).

B.2.1.2.3 Infective dose and transmissibility (in case of pathogenic effect) (Annex IIB 2.2.3)

From open literature, no reports mention infections consecutive to the strain *Paecilomyces fumosoroseus*.

The genus *Paecilomyces* belongs to the group of opportunistic pathogens. They are of inherently low virulence and produce disease only when host resistence to infection is diminished. In people, systemic mycotic infections are seen exclusively in individuals who are immunosuppressed as result of chronic illness, neoplasia, radiotherapy, or chemotherapy.

B.2.1.3 Host specificity range and effects on species other than the target harmful organism (Annex IIB 2.3)

Host range of the fungus :

Paecilomyces fumosoroseus is a fungus found in soil worldwide. It had been reported as a pathogen of many different hosts. The table below based on two references gives a general overview of the variety of hosts of the fungus which is maybe not exhaustive (Smith, 1993; USDA Agricultural Research Service, 1992)

Table B.2.1.3-1 : Host range of *Paecilomyces fumosoroseus*

Order : Family	Pest Species	Substrate
Lepidoptera : Noctuidae	*Spodoptera frugiperda*	
	Spodoptera littoralis	
	Scotia ipsilon	
	Agrotis segetum *A. exclamationis* *Mamestra brassicae* *Autographa gamma* *Spodoptera litura*	cabbage and sugarbeet
	Sesamia nonogrioides	
Tortridicae	*Pammene fasciana*	
Sphingidae	*Marumba quercus*	oak
Pyralidae	*Galleria mellonella*	bees wax
	Ostrinia nubilialis	maize
	Chilo partellus	
	Diaphania hyalinata	melon

Order : Family	Pest Species	Substrate
Plutellidae	*Plutella xylostella*	cabbages
Bombycidae	*Bombyx mori*	silk
Arctiidae	*Utetheisa ornatrix*	
	Spilarctica imparilis	mulberry
	Hyphantria cunea	
Cossidae	*Cossus cadambae*	teak
Carposinidae	*Carposina sasakii*	peach
Gracillariidae	*Conopomporpha cramerella*	cocoa, rambutan and namnan
Diptera : Anthomyiidae	*Delia antiqua*	
Muscidae	*Musca automnalis*	
	Musca domestica	
Calliphoridae	*Calliphora*	
Tachinidae	*Crossocosmia zebina*	parasitoid of *Bombyx mori*
Glossinidae	*Glossinia morsitans*	
Agromyzidae	*Liriomyza trifolii*	
	L. sativae	
Coleoptera :		
Curculionidae	*Otiorrhynchus sulcatus*	
	Sitona lineatus	
Chrysomelidae	*Pyrrhalta luteola* *Spaethiella*	
Scarabaeidae	*Popillia japonica*	
Tenebrioidae	*Tenebrio molitor*	as a trap
Lagriidae	*Lagria vilosa*	
Coccinellidae	*Cycloneda limbifer*	
Scolytidae	*Scolytus multistriatus*	elm
Apionidae	*Cylas formicarius*	sweet potato
Neuroptera : Chrysopidae	*Chrysoperla carnea*	
Hymenoptera : Cephidae	"sawfly"	
Tenthredinidae	*Monophadnus elongatulus*	
Thysanoptera : Thripidae	*Thrips tabaci*	onion
Hemiptera :		

Order : Family	Pest Species	Substrate
Reduviidae	*Rhodnius prolixus*	
Miridae	*Adelphocoris*	
Homoptera :		
Delphacidae	*Nilaparvata lugens*	rice
Pseudococcidae	*Phenococcus solani*	
Aphididae	*Aphis gossypii*	melon
	Myzus persicae	
Aleyrodidae	*Trialeurodes vaporariorum*	greenhouse crops
	Bemisia	watermelon
	Bemisia tabaci	eggplant, gerbera

Epizootics in tropical countries :
Paecilomyces has been found to be one of the most effective pathogens against whitefly under the conditions of lowland tropical areas. It appeared that infection occurred most efficiently during a period of high air humidity. (Dominican Republic) (Peterkin and Hall,1994) Epizootics affecting the whitefly *Bemisia tabaci* have been reported from subtropical areas of the USA, from Mexico, Pakistan, India and Nepal (Lacey et al., 1994) . It is expected to use *Paecilomyces* against whitefly on field crops in Florida and the Caribbean region. (Osborne and Landa, 1992)

Occurrence in cold countries :
In cold countries, the fungus is found in the soil where it can cause infection of the immobile stadia (larvae and pupae) of a wide range of insects.

Potential effects on non-target arthropods :
Due to the fact that the species has been found on a wide range of hosts the possibility that the selected strain *Paecilomyces fumosoroseus* Strain Apoka 97 infects non-target arthropods (beneficial arthropods used in IPM in glasshouse, other non-target organisms) is important. This item will be discussed under point B.8

B.2.1.4 Biology of the micro-organism (Annex IIB 2.4)

- Infective cycle :
A sexual reproductive cycle has not been observed *in P.fumosoroseus*. The life cycle or infective cycle of *Paecilomyces fumosoroseus* is an asexual life cycle.

In general, invasion through the respiratory or alimentary tract has been reported but these are relatively rare invasion sites. The infective unit in most fungi is a spore, usually a conidium. Conidia usually germinate on the cuticle or form appressoria like it is the case of *Paecilomyces fumosoroseus* and then penetrate.
In the case of the formulation PREFERAL, blastospores which are formed in liquid medium by budding of the mycelium, are used as infective units. They are able to germinateon the cuticle and to directly penetrate in the insect body. The further steps of the infective cycle are similar for blastospores and conidia.
Enzymes and mechanical forces are involved. In most cases, but not all, yeast-like fragments of mycelium called hyphal bodies are produced which usually float free and apparently multiply in the haemocoel . After death, or even before, in those strains which are weak toxin producers, normal thread-like mycelium ramifies throughout the internal organs. This continues until the insect is virtually filled with fungus and quite firm to the touch. Conidiophores are then produced which erupt through the cuticle and produce spores on the outside of the insect. With Fungi Imperfecti, conidiophores and conidia are not produced unless the dead insect is in a moist environment.

In this life cycle, aerial mycelium formation in particular is an important cellular differentiation, in that it is closely coordinated with various phenotype characteristics (such as production of antibiotics, pigments, enzymes, earthy

odour and nutrient auxotrophy). (Roberts and Yendol, -)

The infection cycle of sweet potato whitefly, *Bemisia tabaci*, using conidia spores of *Paecilomyces fumosoroseus* Apopka strain 97 is as follows:

1 All stages of the sweet potato whitefly appear susceptible to infection by *Paecilomyces fumosoroseus* Apopka strain 97.

2 Conidia, the infectious stage of the fungus, passively contact and adhere to the cuticle of the juvenile whitefly or whitefly egg. Within 12 hours, the conidia germinate, as evidenced by the presence of a germ tube .

3 During the next 12-48 hours, the fungus grows extensively over the surface of the insect and also penetrates the cuticle of the insect via mechanical and enzymatic processes . Once inside the host, the fungus can utilize the insect body tissues as a nutrient source.

4 At some point 24-48 hours after germination and during fungal proliferation within the insect body, the insect dies . Upon nutrient depletion, the fungus exits the body and sporulates, producing new conidia .

Mechanism of how spores (conidia or blastospores) of *Paecilomyces fumosoroseus* penetrate into the body of the insect is not conclusively established. However, it is speculated that *Paeciloymyces fumosoroseus*, as many other entomogenous fungi, may produce cellulolytic enzymes such as chitinases, amylases, and proteases which can degrade the wall of the insect bodies.
(Eyal, 1994a)

The conidium/blastospore or asexual spore is uninucleated or multinucleated and can be either haploid or diploid. Multinucleated conidia can be heterokaryotic.
Fusions occur between hyphae carrying nuclei of like or unlike genotype. In the latter case, they give heterokaryotic cells with nuclei of different genotype in a common cytoplasm. Macroscopically, the heterokaryon appears as a mosaic of conidial heads of 2 colours.

Parasexual cycle :
One of the most vital functions performed by the sexual cycle is genetic recombination.
Parasexuality essentially involves recombination without sex. A parasexual cycle seems also to exist for *P. fumosoroseus*.
The parasexual cycle has in general three stages:
1) fusion of adjacent somatic hyphae, and exchange of nuclei, establishing a heterokaryon.
2) Fusion of different nuclei in the vegetative hyphae, to form somatic diploids.
3) Somatic recombination (mitotic crossing over)
4) Non-meiotic reduction of the altered nuclei to the haploid condition.
This sequence of events is rare, happening in fewer than one conidium in a million, but the number of conidia produced by most conidial anamorphs is astronomical, so parasexuality is a practical means of producing genetic variation.
The parasexual cycle of *P. fumosoroseus* is probably not the same as the typical cycle described above since Riba and Ravelojoana (1984) were unable to discriminate between the heterokaryons and the diploids. This is probably due to the fact that the hyphal cells contain only two nuclei resulting in instability in the heterocaryotic stage.
Moreover, under some growth conditions, a few mutant strains of *P. fumosoroseus* were able to mate in the presence or in the absence of host insects and, following hybridization, presumptive diploids or haploid recombinants could have been generated. Competition between the latter and the mutant parental strains during the pathogenesis in the host insects was observed.
Auxotrophic recombinants were identified by recombination in the same hybrid of two markers from two parents. Only a few of the possible recombinant classes were obtained from the diploids generated by hybridization of diauxotrophic mutants, but this probably was the result of the small numbers of haploid sector analyzed. It is more

probable that some of the prototrophs must be considered as haploid recombinants. It is posssible to obtain diploids or mitotic recombinants in *P. fumosoroseus* (Riba and Ravelojoana, 1984).

Occurrence of resting stages :
Viable inoculum is maintained in the environment by the production of conidia or resting spores in or on the host. See point B.7 Fate and behaviour in the environment.

B.2.1.5 Infectivity. Dispersal and colonization ability (Annex IIB 2.5)

- *Environmental factors affecting the fungal life cycle of P. fumosoroseus:*

1. Effect of temperature on Fungus Growth Development Index (FGDI) (Landa et al., 1994)

Bioassay protocol:
1 Nymph of SLWF (*Silverleaf Whitefly* nymphs) or GHWF (*Trialeurodes vaporariorum*) was placed in the middle of a conidial drop (approx. 0.003 ml per drop). A total of 250 nymphs were tested /fungus. Controls were performed in parallel. Each sample was adjusted to a final concentration of 1.0×10^7 conidia/ml.
A rating system was used to assess the degree of fungal development on the host insect. The rating index is the "Fungus Growth Development Index" or FGDI. Three FGDI values are considered to be the most critical values during the assay (0.5, 1.5 and 2.5). Each of these values represents the beginning of a growth phase of the fungal life cycle. The initiation of the cycle is manifested by a FGDI value of 0.5. This stage represents the first sign of viability of the conidia. The colonization of the host with the fungus is defined by an FGDI value of 1.5. This phase of the fungal development is irreversible and the host insects do not recover from infection. An FGDI value of 2.5 represents the initial sporulation of the mycelium on the nymphs.
Findings :
Paecilomyces fumosoroseus Apopka Strain 97 is an isolate that will rapidly develop on whitefly nymphs and can complete its life cycle in less than 120 h. It was evident that nymphs induce fungal development.
Faster development and a rapid increase in FGDI values were recorded at 30°C. Colonization of the host by the fungus (FGDI=1.5) was obtained within 48 h and the beginning of sporulation (FGDI higher than 2.5) was noted by 96 h.
A slow increasee in FGDI values occurred when *Paecilomyces fumosoroseus* Apopka Strain 97 was assayed at temperatures below 20°C.
At 18°C, the FGDI value of 2.71 was obtained only at 168 h, demonstrating that this strain can complete its life cycle at this temperature. However, at this temperature, *Paecilomyces fumosoroseus* Apopka Strain 97 reached the critical value of 1.5 after 96 h.
Extremely slow development was typical when *Paecilomyces fumosoroseus* Apopka Strain 97 was tested at 12°C; however, the FGDI value increased within the period of the assay (1.33 at 168 h) (Table B.2.1.5-1)

Table B.2.1.5-1 : FGDI of *Paecilomyces fumosoroseus* Apopka Strain 97 against Silverleaf Whitefly at constant Relative Humidity.

	Constant temperature (°C)			
Hours	**12**	**18**	**24**	**30**
24	0.00 ± 0.00	0.11± 0.13	0.81 ± 0.24	0.63 ± 0.18
48	0.13 ± 0.16	0.62± 0.23	1.35± 0.23	1.56± 0.14
72	0.32± 0.21	1.12 ± 0.13	1.81± 0.11	2.12 ± 0.22
96	0.54± 0.28	1.45 ± 0.32	2.02± 0.21	2.52 ± 0.09
120	0.81 ± 0.33	1.91 ± 0.39	2.41 ± 0.2	2.83 ± 0.09

144	1.06 ± 0.27	2.42± 0.22	2.72± 0.13	3.00 ± 0.00
168	1.33± 0.27	2.71± 0.29	3.00 ± 0.00	3.00± 0.00

Germination and growth of PFR 97 WDG-Apopka under temperatures above 30°C (Landa, 1996)
Findings:
- at 25°C : very good germination and development based on evaluation using germination index (FGDI) values. Viability of conidia in standard conditions is the confirmation of generally good viability of tested population.
- at 32°C : Conidia of PFR 97 germinate fairly good. When compared with control set, some slow down of both, either of germination (%) and of FGDI is visible. However on day 4 there were newly born conidia visible (FGDI = 2.5 and higher).
- at 33 and 35°C : none of germinating conidia was recorded within first four days of experiment. Consequently, no FGDI higher than 0 was found. 1600 Conidia for each temperature were observed and assessed within 4 days. (Table B.2.1.5-3)

Table B.2.1.5-2 : Germination and growth of *Paecilomyces fumosoroseus* Apopka Strain 97 under different temperatures.

Day of assay	**Assessed parameter**	**const.temp. 25°C**	**const.temp. 32°C**	**const.temp. 33°C**	**const.temp. 35°C**
1	germination (%) FGDI	97.32 1.14	66.12 0.21	0 0	0 0
2	germination (%) FGDI	98.52 2.78	89.33 1.86	0 0	0 0
3	germination (%) FGDI	- 3.00	88.17 2.19	0 0	0 0
4	germination (%) FGDI	- -	- 2.49	0 0	0 0

No indication on the quality of the information is provided . These results confirm other informations on the development temperatures of *P. fumosoroseus*

In another study, the influence of temperature on the *in vitro* growth of *Paecilomyces fumosoroseus* was evaluated at 10 temperature levels from 8 to 37°C in the dark. (Fargues, 1992)
Findings :
10 strains of *Paecilomyces fumosoroseus* isolated on different insect species in France were grown in vitro to determine growth rates and ranges of growth at 10 temperatures from 8 to 37 °C in the dark. Growth rates (mm/day) were used as the main growth parameter to evaluate the influence of temperature.

Table B.2.1.5-3 : Growth rates of *Paecilomyces fumosoroseus* on solid growth medium - (average for the 10 strains *)

	Growth temperatures (°C)								
	8	**11**	**15**	**20**	**25**	**28**	**30**	**32**	**35**
growth rate	0	1.36	2.24	3.83	4.11	2.45	0.57	0	0

(mm/day)									

(*) : Results for each strain are reported in the original publication.

No fungus growth is observed at 8°C. Growth rate increases from 11 °C up to a maximum at the optimum rate (20-25°C). Growth rates are decreasing rapidly at higher temperatures (25-30°C). At 32 and 35°C no fungus growth is observed. The growth curves for the 10 studied strains were very homogeneous.

2. Effect of relative humidity on Fungus Growth Development Index (FGDI) (Landa et al., 1994)

Decrease in the relative humidity resulted in significant inhibition in activation of the fungus on the insect host. The initial activation (swelling) of conidia was observed at 168 h at 85% relative humidity (FGDI 0.5).
At 80% relative humidity, *P.fumosoroseus* did not initiate any development. When the assay was conducted with humidity levels alternating between an optimal relative humidity of 100% and suboptimal relative humidity of 90%, and the period of optimal humidity was maintained longer than 12 h, higher FGDI were obtained than when the optimal humidity cycles of less than 12 h were tested. The FGDI values obtained were significantly lower and identical to FGDI values obtained when maintained at a constant 90% relative humidity.
To obtain faster development of the fungus, 100% relative humidity was needed as soon as the fungus was applied and must be maintained for at least 12 h. Any fluctuation in relative humidity during the first 24-h period after application resulted in a lower rate of fungal development.

3. Effect of light on fungal development:

- Light also affects some aspects of fungal development, particularly the last phase of the infection cycle-conidiogenesis. A significant increase in the production of conidia was observed when cultures of *P.fumosoroseus* were exposed to fluorescent light (Osborne and Landa, 1992).

- Inactivation of conidia of *Paecilomyces fumosoroseus* by near-ultraviolet (UVB and UVA) and visible light:
The detrimental effects of solar radiation, especially the ultraviolet waveband, on quiescent conidia of *P.fumosoroseus* were investigated
Conidia were irradiated by a high-intensity source, which emitted a continuous spectrum from 270 to 1100 nm and which was equipped with long-pass filters to block short wavelengths below 280, 295, 320, or 400 nm. Detrimental effects of light depended on irradiance in the shortest wavelengths. The UVB (280-320 and 295-320 nm) appeared to be the most detrimental part of natural radiation, although UVA (320-400 nm) was also harmful. Visible and near infrared radiations were less harmful than UV. Irradiance of the UVB waveband should be considered as the pertinent factor for the detrimental effects of sunlight on the persistence of conidia of entomopathogenic fungi in insolated environments .
Nevertheless, the results provided by irradiation tests with *P.fumosoroseus* conidia cannot be directly extrapolated to natural conditions. Under field conditions, persistence of hyphomycetous conidia at the top of the canopy shows a slower decay than those obtained in artificial irradiation. It is not surprising that photic effects can be overestimated through artificial irradiation , as the spectral irradiance of artificial and natural radiation differs in the lowest UVB wavelengths and the incidence angle of the sun's rays on the foliage surface is very variable. Almost no radiation with wavelength under 295 nm reaches the earth's surface. In greenhouse environments, however, cover material drastically changes the radiation spectrum, particularly in blocking most UVB radiation. The susceptibility of *P.fumosoroseus* inocula to UV radiation suggests the use of greenhouse covers blocking UVA and UVB radiation to improve the persistence of mycoinsecticides (Fargues et al., 1997).

4. Other factors affecting the colonization ability of *P. fumosoroseus*:

- Effect of nutrients and surfactants on FGDI:
The influence of various commercial adjuvants and nutrients on the development and growth of PFR 97 on SLWF was generally an inducing effect.
Some of the surfactants tested induced faster development of PFR 97 (Foam Buster, Tween 80, Big Wet) while others appeared to have little impact when compared with the control.

Most nutrients induced and increased the FGDI values and evidently supported faster establishment of fungus on the host. The additives that decrease the FGDI value indicate that such material should not be used with the fungus (e.g., Leaf Act) (Landa et al, 1992).

5. Sensitivity of *P. fumosoroseus* to fungicides:
See point B.2.2.8.3

6. Persistence in soil:
See point B.7 Fate and behaviour in the environment.

B.2.1.6 Relationships to known plant or animal pathogens (Annex IIB 2.6)

Some species of the genus *Paecilomyces* were found to be pathogenic to humans and/or animals. However, no pathogenic effects were related to *Paecilomyces fumosoroseus*.

One case, reported in the literature as consecutive to an infection by *Paecilomyces fumosoroseus*, was in fact a misidentification of the isolated strain :
A cat was euthanatized following a mycotic infection. Fungal cultures of the aspirates and debrided tissues resulted in isolation of *Paecilomyces fumosoroseus*. Postmortem examination demonstrated nasal lesion , paw lesion and enlarged liver containing white nodules (Elliott et al., 1984). The results of Dr. Samson's comparison indicate that the authors of this paper misidentified the isolated strain from the cat. All of the original vials including the ATCC depository have been found to contain *Paecilomyces lilacinus,* a known pathogenic strain (notifier clarification).

Paecilomyces species are saprophytic fungi and have been reported to cause various types of infection, such as cutaneous mycosis, endocarditis and oculomycosis in immunosuppresssed patients. *Paecilomyces* species are widely distributed throughout the environment. They are frequently detected in soil samples, decomposing vegetation, and as airborne contaminants.

Despite its ubiquity, *Paecilomyces lilacinus* remains an uncommon cause of fungal infections in humans. The majority of case reports describe ocular infections. Others have implicated *Paecilomyces lilacinus* as a cause of dermatologic infection, sinusitis, pulmonary mycosis, an abdominal wall abscess, and fungemia related to an indwelling catheter. Numerous strains of *Paecilomyces lilacinus* are capable of producing potent mycotoxins. Leucinostatins derived from cultures of the organism are highly toxic to experimental animals and appear to have had a direct impact on the tissue inflammatory response. Inoculation of leucinostatin directly into cornea of a rabbit resulted in abscess formation. These findings suggest that toxin production by *Paecilomyces lilacinus* may be an important virulence factor (Westenfeld et al., 1996). Paecilotoxins are neutral straight peptide toxins, which include unsaturated fatty acid in the N- terminal group and amine in the C-terminal. Paecilotoxins are also characterized as having a high content of peculiar amino acid a-aminoisobutyric acid. Biological studies of the toxins showed that they have strong uncoupling activity against rat liver mitochondria as well as antimicrobial activity. Rate of the toxin producing-strains increased with incubation time and after 15 days of culture most of the strains tested produced the toxins. An alkaline medium is more appropriate than a neutral or acid one for the production of paecilotoxins. Since paecilotoxin production was confirmed in most strains of *P. lilacinus*, this character might be used as chemotaxonomical index for this species (Mikami et al, 1989).
Conjunctivitis appeared as early as one day after the intracorneal inoculation of the spore suspension as well as the direct administration of the mycotoxin. Corneal opacity was observed soon after the administration of leucinostatin, but there was three days' lag in the case of the inoculation of the fungus spores. The important role of leucinostatin in the inflammatory response of the tissue invaded by the fungus was suggested (Mikami et al., 1984).

Soft tissue infection was reported in a 8-year old child . Infection was insidious, and minor heel pain and fevers occurred only on the day interferon-gamma was injected. Soft tissue biopsy showed hyphal elements, and *Paecilomyces variotti* grew in culture (Williamson et al., 1992).
Paecilomyces most probably *variotti* was isolated from laboratory rats. In about 90% of cases the organism was localized in the respiratory tract and there are some other findings favouring the hypothesis of an airborne infection. During the 12 year observation period no consequences for the health of rats in one closed infected SPF breeding unit could be observed (Kunstyr et al., 1997).

B.2.1.7 Genetic stability and factors affecting it (Annex IIB 2.7)

Genetic stability during manufacturing process :
During the manufacturing process of PFR-MUP the same basic material is used all the time. Consequently the chance of genetic changes may be called extremely slim. In order to maintain the genetic stability and pathogenicity (degree of virulence) of PFR-MUP during the production process, 100 recipients of the first culture of the original isolate are carefully kept in the Thermo-Trilogy laboratories as a mother stock or master culture. This stock is derived from one single culture of the original fungus on agar. Out of 1 such bottle 100 new ones are cultivated. For the production of each batch, each time one of these bottles is used for the inoculaton of the fermentor. This means that 10 000 fermentations may be carried out with only two intermediate steps on agar. This shows that the fungus is cultivated only a minimal number of times during the production process. Thus a standardized product is obtained, derived from the same parent stock, and therefore the characteristics and stability of *Paecilomyces fumosoroseus* strain Apopka 97 have remained constant throughout the production process.

Genetic stability under natural conditions :
Under some growth conditions, a few mutant strains of *P. fumosoroseus* were able to mate in the presence or in the absence of host insects and, following hybridization, presumptive diploids or haploid recombinants could have been generated. Competition between the latter and the mutant parental strains during the pathogenesis in the host insects was observed (Riba and Ravelojoana, 1984).
The mechanism of loss and reacquisition of virulence is unknown, but its occurrence with single spored cultures indicates it is not accomplished by selection of specific genotypes such as could occur with mixed cultures.
Other factors which may account for reduced virulence include heterokaryosis and /or somatic recombination from the anastomosis of hyphae of different genotypes, and the amount of saprobic growth the fungus has undergone in the environment prior encountering insects. (Roberts and Yendol, -)
Heterokaryosis in *Paecilomyces fumosoroseus* may occur, but at a very low frequency. Heterokaryotic colonies are unstable, and have a very low growth, evenly on selected grow medium. This instability is not an hindrance to obtaining stable mitotic recombinants. The mitotic recombinant conidia are haploid (Riba, 1978).

B.2.1.8 Information on the production of toxins (Annex IIB 2.8)

Bauverolides are not present in the active substance (PFR-MUP) or in the formulation PREFERAL WG (see B.1.1.4.2).

B.2.1.9 Resistance to antibiotics (Annex IIB 2.9)

No information required in the case of a fungus (Annex point specific to bacteria)

Paecilomyces fumosoroseus is resistant to the antibiotics streptomycin sulfate and chlortetracycline hydrochloride which are used to prepare a selective medium for the growth of the fungus (Hunsberger A., 1993)

B.2.1.10 Summary (Annex IIB 2.2.2)

Paecilomyces fumosoroseus causes infections of all stages (eggs, immature stages and adults) of greenhouse whitefly (*Trialeurodes vaporariorum*).
PFR 97 strain is an isolate that will rapidly develop on whitefly nymphs and can complete its cycle in less than 120h. Spores (conidia and blastospores) of the fungus germinate on the insect after the application. The fungus penetrates into the body of the insect where it develops, causing the death after 7-10 days.
Dense hyphal masses may form in the host and mechanically disrupt the host's internal organs or pervasive hyphal activity may initiate tissue necrosis and loss of function. In high relative moisture conditions, the fungus can grow outside of the insect and produce new spores.

Relative humidity plays the most important role among abiotic factors affecting the infection cycle of the fungus. Germination of conidia is retarded if relative humidity declines under 98% and is usually impaired at humidities lower than 90%. Under adverse conditions, the fungus may stay dormant inside the dead host for several weeks and become active if conditions are suitable.
It seems therefore critical to have at least a short period of high humidity during the initiation of the infection process (conidial attachement to the host surface, conidial swelling, germination, penetration of the host cuticle). *P.fumosoroseus* requires for this portion of the infection cycle, a relative humidity above 95%.
Temperature seems to be less of a limiting factor. The fungus grows and multiplies at temperatures between 15 and 30°C with colony growth optimal between 23 and 25°C. Germination of conidia and growth of mycelium decline above 25°C and cease above 32°C.
At 19°C in soil, the infection potential of *Paecilomyces fumosoroseus* was substantially degraded after 6 months incubation. Isolates of *Paecilomyces fumosoroseus* are of low tolerance as their growth is limited between 11 and 30°C. The UVB appeared to be the most detrimental part of the natural radiation ; visible and near infrared radiations were less harmful.
Paecilomyces sp are widely distributed throughout the environment and fungal infections in humans were reported with the *lilacinus* and *variotii* species. No reports were found where the *fumosoroseus* species was the causative agent.
The chances of genetic changes are slim. Secondary metabolites were not detected in the PFR97 WDG- Apopka as their production needs special growth phase or special growth conditions.

B.2.2 Technical properties of the preparation PREFERAL WG (Annex IIIB 2)

Study	Guidelines and GLP	Findings	Evaluation and conclusions	References
B.2.2.1 Appearance (colour, odour, physical state) (IIIB 2.1)	ASTM D1535	colour : tan; 10 YR 6/4 by Munsell Color System	Acceptable	Eyal, 1994(a)
	-	odour : wheat bran	Acceptable	Eyal, 1994(a)
	-	Physical state : solid	Acceptable	Eyal, 1994(a)
	ASTM D941, ASTM D1298, CIPAC MT 3, OECD 109	Specific gravity : 0.445 g/ml	Acceptable	Eyal, 1994(a)
B.2.2.2.1 Storage stability and shelf-life - Effects of light, temperature and humidity (IIIB 2.2.1)	CIPAC MT46, OECD 113	influence of temperature was studied : - storage at 4°C for 120 days showed no significant decrease in CFU/g - storage at higher temperatures (25°C, 30°C and 40°C) on the other hand already showed a significant decrease in CFU/g after 30 days ⇒ storage under refrigeration is required to ensure maximum shelf life Applicant states that a shelf life of six months can be easily achieved when the product is stored at 4°C.	Acceptable, but additional information concerning the effects of light and humidity on the product stability is required. Additional testing to determine if the product will be stable for more than one year when stored under refrigeration is ongoing.	Eyal, 1994(a)
B.2.2.2.2 Storage stability and shelf-life - Effect of other factors (air, packaging,...) (IIIB 2.2.2)		no information provided	Additional information required	
B.2.2.3 Explosivity and oxidizing			Not required (formulation containing food and/or	

Study	Guidelines and GLP	Findings	Evaluation and conclusions	References
properties (Annex IIIB 2.3)			pharmaceutical grade inert materials + a microbial active substance that is not an oxidizer or a reducing agent, nor presents an explodability hazard)	
B.2.2.4 Flash point and other indication of flammability or spontaneous ignition (Annex IIIB 2.4)			Not required (formulation containing food and/or pharmaceutical grade inert materials + a microbial active substance that is not flammable)	
B.2.2.5 Acidity, alkalinity, pH (Annex IIIB 2.5)	ASTM E70	pH = 6.4	Acceptable	Eyal, 1994(a)
B.2.2.6 Viscosity and surface tension (Annex IIIB 2.6)			Not applicable (formulation is not a liquid)	
B.2.2.7.1 Wettability (IIIB 2.7.1)	CIPAC MT 53.3.2	wetting time = 22 seconds (with swirling)	Acceptable	Benoit, 1995
B.2.2.7.2 Persistent foaming (IIIB 2.7.2)	CIPAC MT 47	0 ml foam after 0 seconds	Acceptable	Benoit, 1995
B.2.2.7.3 Suspensibility and suspension stability (IIIB 2.7.3)	CIPAC MT 168	suspension stability = 50% spontaneity of dispersion was not determined	Acceptable, provided that appropriate instructions (e.g. continuous agitation) are mentioned on the label.	Benoit, 1995

Study	Guidelines and GLP	Findings	Evaluation and conclusions	References
B.2.2.7.4 Wet sieve test and dry sieve test (IIIB 2.7.4)	CIPAC MT 59.3	wet sieve residue on 0.100 mm sieve = 100% (blockage)	Not acceptable : when applying the formulation with the usual spraying equipment, blockage of nozzles seems unavoidable.	Benoit, 1995
	CIPAC MT 167	wet sieve residue on 0.075 mm sieve = 31%	Not acceptable : when applying the formulation with the usual spraying equipment, blockage of nozzles seems unavoidable.	
B.2.2.7.5.1 Particle size distribution (powders) - nominal size range of granules (IIIB 2.7.5.1)	CIPAC MT 58	residue on 1 mm sieve : 0% residue on 0.5 mm sieve : 0% residue on 0.1 mm sieve : 98% residue on 0.075 mm sieve : 1% residue in receiving pan : 1%	Acceptable	Benoit, 1995
B.2.2.7.5.2 Dust content (granular preparations) (IIIB 2.7.5.2)		no information provided	Additional information required	
B.2.2.7.5.3 Friability and attrition characteristics (granules) (IIIB 2.7.5.3)			Not applicable (no internationally agreed methods available at the time)	
B.2.2.7.6.1 Emulsifiability, re-emulsifiability, emulsion stability of preparations which form emulsions (IIIB 2.7.6.1)			Not applicable (formulation does not form emulsions)	
B.2.2.7.6.2 Stability of dilute emulsion and			Not applicable (formulation is not an emulsion)	

Study	Guidelines and GLP	Findings	Evaluation and conclusions	References
of preparations which are emulsions (IIIB 2.7.6.2)			not an emulsion)	
B.2.2.7.7.1 Flowability (granular formulations) (IIIB 2.7.7.1)	CIPAC MT 172	Residue on 4 mm sieve : - initially : 73% - after 5 liftings : 14% - after 20 liftings : 2%		
B.2.2.7.7.2 Pourability (suspensions) (IIIB 2.7.7.2)			Not applicable (formulation is not a suspension)	
B.2.2.7.7.3 Dustability (dustable powders) (IIIB 2.7.7.3)			Not applicable (formulation is not a dustable powder)	
B.2.2.8.1 Physical compatibility of tank mixes (IIIB 2.8.1)			PREFERAL must not be used in tank mix	
B.2.2.8.2 Chemical compatibility of tank mixes (IIIB 2.8.2)			PREFERAL must not be used in tank mix	
B.2.2.8.3 Biological compatibility of tank mixes (IIIB 2.8.3)		The effect of 30 chemical pesticides on the *in vitro* growth of PFR was determined. Only four pesticides produced a zone of inhibition (Captan, Prochloraz, Terraguard and Manzate 200) and Captan was the only fungicide that limited the spread of PFR.	Acceptable	Eyal, 1994(b)
B.2.2.9 Adherence and distribution to seeds (IIIB 2.9)			Not applicable (formulation is not intended for seed treatment)	

ANNEX B

Paecilomyces fumosoroseus Strain Apopka 97

B.3 Data on application and further data

B.3.1 Further information on the organism (Annex IIB 3)

B.3.1.1 Function (Annex IIB 3.1)

Insecticide

B.3.1.2 Field of use envisaged (Annex IIB 3.2)

Insecticide for the control of whitefly *(Trialeurodes vaporariorum)* in greenhouse vegetables (cucumber and tomato). The fungus will be used to support the biological control of whiteflies with the parasitoid *Encarsia formosa* and other natural enemies like the mirid bug *Macrolophus caliginosus* (i.e. the fungus will be used when whiteflies infestation cannot be controlled by the natural enemies alone)

This insecticide would be used as 'spot treatment' on the places where a high whiteflies infestation would occur. One application per growing season would be the most probable scenario.

B.3.1.3 Crops or products protected or treated (Annex IIB 3.3)

The table below is a short overview of the intended uses of PREFERAL WG. A more detailed description of the application method is given under point B.3.2 .

Table B.3.1.3-1 : Summary table of the intended uses of PREFERAL WG

Crop **Pest**	**Country**	**Maximum rate per application (kg /ha)**	**Maximum rate per season (kg /ha)**	**Spray concentration (g/hl)**	**Maximum number of applications per season**	**spray interval in days**	**Pre-harvest interval in days**
Tomato (glasshouse) *Trialeurodes vaporariorum*	all Europe	1-3 kg formulation/ha	3-9 kg formulation/ha	100 g formulation/hl	1-3	7 days	*
Cucumber (glasshouse) *Trialeurodes vaporariorum*	all Europe	1-3 kg formulation/ha	3-9 kg formulation/ha	100 g formulation/hl	1-3	7 days	*

* : PHI determination is not relevant. This issue is further discussed under point B.6 - Residues on treated crops

(The notifier is aware of the fact that the product cannot be used in hot and dry environmental conditions)

B.3.1.4 Method of production (Annex IIB 3.4)

Confidential information : See Annex C

B.3.1.5 information on the occurrence or possible occurrence of the development of resistance (Annex IIB 3.5)

The notifier is aware of the fact that development of resistance could occur and is developing anti-resistance strategies (use of PREFERAL WG in combination with beneficial arthropods and chemicals harmless to beneficial arthropods, limitation of the number of applications per season)

B.3.1.6 Methods to prevent loss of virulence of seed stock and micro-organism (Annex IIB 3.6)

Research of other entomopathogenic fungi has raised the suspicion that the frequent passing of an entomopathogenic fungus on an artificial medium may lead to loss of virulence. In order to prevent the loss of virulency, 100 recipients of the first culture of the original isolate are carefully kept in the Thermo-Trilogy laboratories as a mother stock culture. Out of 1 such vial 100 new ones are cultivated. For the production of each fermentation batch, one of these vials is used for the inoculaton of the fermentor. Therefore, an identical fungus is cultivated during the production process. Thus a standardized product is obtained, derived from the same mother stock.

B.3.1.7 Recommended methods and precautions during handling, storage, transport or fire (Annex IIB 3.7)

This information is presented under the form of a safety data sheet pursuant to Article 27 of Council Directive 67/548/EEC. See Appendix A : Material Safety Data Sheets .

B.3.1.8 Procedures for destruction or decontamination (Annex IIB 3.8)

P. fumosoroseus is known mainly as an insect parasite with a wide host range and a worldwide distribution from Europe to Africa and Asia. *Paecilomyces fumosoroseus* was not found to be pathogenic to humans and/or animals.

Therefore, due to its natural occurrence and its ubiquitous distribution, the absence of pathogenicity to humans, procedures of destruction are not necessary.

B.3.1.9 Measures in case of an accident (Annex IIB 3.9)

Due to its natural occurrence, the absence of pathogenicity to humans, its ubiquitous distribution, accidents are unlikely to occur.

B.3.2 Data on application of preparation PREFERAL WG (Annex IIIB 3)

B.3.2.1 Field of use envisaged (Annex IIIB 3.1)

Insecticide for the control of whitefly *(Trialeurodes vaporariorum)* in greenhouse vegetables (cucumber and tomato). The fungus will be used to support the biological control of whiteflies with the parasitoid *Encarsia formosa* and other natural enemies like the mirid bug *Macrolophus caliginosus.* (i.e. the fungus will be used when whiteflies infestation cannot be controlled by the natural enemies alone)

This insecticide would be used as 'spot treatment' on the places where a high whiteflies infestation would occur. One application per growing season would be the most probable scenario.

B.3.2.2 Mode of action (Annex IIIB 3.2)

Blastospores of the fungus germinate on the insect after the application. The fungus penetrates into the body of the insect where it develops, causing the death after 7-10 days.
In high relative humidity conditions, the fungus can grow outside of the insect and produce new spores.

B.3.2.3 Detail of intended use (Annex IIIB 3.3)

See point B.3.1.3.

B.3.2.4 Application rate (Annex IIIB 3.4)

See point B.3.1.3.

B.3.2.5 Content of micro-organism in material used (Annex IIIB 3.5)

	Concentration in CFU	Concentration in blastospores and mycelia
'Technical' *Paecilomyces*, PFR-MUP, fermentation broth	at least 1. 10^8 CFU/ml	70 g/l
PREFERAL WG	at least 2.10^9 CFU/g	200 g/kg
Spray concentration (100 g PREFERAL WG/hl)	2.10^9 CFU/l 2.10^{11} CFU/hl	0.2 g/l
Application rate per ha, assuming that 1000-3000 l/ha are sprayed	$2..10^{12}$ - 6.10^{12} CFU/ha	200-600 g/ha

B.3.2.6 Method of application (Annex IIIB 3.6)

The treatment has to reach the under side of the leaves for a good efficacy. 1000-3000 l spray solution/ha depending on the height of the crop is therefore recommended.
- High relative air humidity (>80% , during 12 hours) is necessary for the growth of the fungus. Watering the greenhouse in the evening is recommended.
- Optimum growth temperature is 20-28°C.

B.3.2.7 Number and timing of application and duration of protection (Annex IIIB 3.7)

The product must be applied when the first whiteflies appear. On cucumber and tomato treatments must be realised 1-3 times with a 7 day interval.

B.3.2.8 Necessary waiting period or other precautions to avoid phytopathogenic effects on succeeding crops (Annex IIIB 3.8)

Not required. The fungus was only found on infected insects. No indication on phytopathogenic effects is reported.

B.3.2.9 Proposed instruction for use (Annex IIIB 3.9)

See label - Appendix A

B.3.3 Further information on the preparation (Annex IIIB 4)

B.3.3.1 Packaging (type, materials, size, etc.) and compatibility of the preparation with proposed packaging materials (Annex IIIB 4.1)

500 g capacity cardboard packaging

Information of the compatibility of the preparation with the packaging is not required due to the nature of the formulation (organic material, food grade ingredients)

B.3.3.2 Procedures for cleaning application equipment (Annex IIIB 4.2)

It is stated that the spraying equipment must be thoroughly cleaned before application of PREFERAL WG.
No specific procedures are mentioned for cleaning application equipment after use.

B.3.3.3 Re-entry periods, necessary waiting periods or other precautions to protect man, livestock and the environment (Annex IIIB 4.3)

Not required. See point B.6 Residue on treated crops.

B.3.3.4 Recommended methods and precautions concerning handling, storage, transport (Annex IIIB 4.4)

Store packagings in a dry, cool place (2-6°C). Shelf-life of 6 months at 4°C can be achieved.

B.3.3.5 Measures in the case of an accident (Annex IIIB 4.5)

No specific measures are recommended. The formulation contains only non hazardous ingredients (non pathogenic fungus, food grade ingredients)

B.3.3.6 Procedures for destruction or decontamination of the preparation and its packaging (Annex IIIB 4.6)

Paecilomyces fumosoroseus is mass- produced by a fermentation procedure which gives rise to a majority of blastospores. These yeast-like cells , when dried , are short lived organisms.

B.3.3.6.1 Controlled incineration (Annex IIIB 4.6.1)

No specific measures are recommended. The formulation contains only non hazardous ingredients (non pathogenic fungus, food grade ingredients)

B.3.3.6.2 Other methods of destruction (Annex IIIB 4.6.2)

No specific measures are recommended. The formulation contains only non hazardous ingredients (non pathogenic fungus, food grade ingredients)

ANNEX B

Paecilomyces fumosoroseus Strain Apopka 97

Appendix A - Material Safety Data Sheets
Formulation label

ANNEX B

Paecilomyces fumosoroseus Strain Apopka 97

B.4 Analytical methods

B.4 Analytical methods

B.4.1 Analytical methods - micro-organism (Annex IIB 4)

B.4.1.1 Methods for the identification of the micro-organism (Annex IIB 4.1)

PFR-MUP Manufacturing and Analysis of Product (Eyal J., 1994)

Standard Operating Procedures to control the quality of the batches and identify the micro-organism are available. These SOPs are used routinely by the notifier to control the quality of his products.

928-4774-RM Raw Material Specifications
928-4775-SM Preparation of PFR Master and Working Stock Vials
928-4775-VB Assay for Determining Viability of Stock Culture
928-4775-SP Determination of Purity of PFR Culture and Fermentation Broth and Fermentation Samples
928-4775-IP Inoculum Flask Preparation
928-4775-FI Inoculum Seed Preparation in 150 l Fermentor
928-4775-FP Fermentation in 1,500 or 4,000 l Fermentor
928-4775-PH Assay for pH and Probe Standardization
928-4775-DW Assay for Dry Weight
928-4775-SP Assay for Determining Spore/ml
928-4775-CFU Assay for Determining CFU/ml

Table B.4.1.1-1 :Sampling/Quality Control/Analytical Methods During Fermentation

ITEM	SOP	METHOD
Viability of Stock Culture	928-4775-VB	Plating on agar
Spore Count/ml	928-4775-SM	Hemacytometry
CFU/ml	928-4775-CFU	Plating
Cell Mass	928-4775-DW	Dry Weight
Contamination	928-4775-SP	Plating on agars
Oxygen Concentration	928-4775-FI	Monitoring Continuous on-line
pH	928-4775-PH	Continuous on-line probe

Conclusions :
Techniques used to ensure a uniform product and assay methods for its standardization as well as information on quality control measures during production process were provided.
SOP to determine the presence of bacterial contamination was submitted.

B.4.1.2 Methods to determine and quantifiy viable and non-viable residues of active organism, secondary metabolites and degradation products in or on treated products, foodstuffs, feedingstuffs, animal and human body fluids and tissues, soil, water and air, where relevant (Annex IIB 4.2)

B.4.1.2.1 Methods to determine and quantify residue in foodstuffs

Methods to determine and quantify residue were not required due to the absence of residue (fungus and/or toxins) on the fruits (cucumbers and tomatoes). This issue is further discussed under point B.6 - Residues on treated products

B.4.1.2.2 Methods to determine and quantify residue in soil, water and air

A selective medium for the detection of *Paecilomyces fumosoroseus* in greenhouse soil and its potential as a microbial control agent of *Diaprepes abbreviatus* larvae in containerized citrus (Hunsberger, 1993)
Principle of the method :
- A selective medium for the quantification of *Paecilomyces fumosoroseus* isolated from greenhouse soil has been developed. Three growing media (Pasteur's complete medium MC, potato dextrose agar PDA, and oatmeal agar OMA) were selected as basal enrichments for the development of selective medium for *Paecilomyces fumosoroseus*. Combinations of fungicides, salts and antibiotics (dodine, benomyl, NaCl, pentachloronitrobenzene, streptomycin sulfate and chlortetracycline hydrochloride) were tested as antimicrobial agents and for their impact on *Paecilomyces fumosoroseus.*
- The final medium includes 0.4 g dodine, 1 ml Tergitol, 0.1 g streptomycin sulfate, and 0.05 g chlortetracycline hydrochloride/l PDA.
Selective plates could be accurately read for *P. fumosoroseus* colonies, and most fungal and all bacterial contaminants were suppressed. All selective plates could be read on day 7 post-inoculation for *P. fumosoroseus* colonies. By this time, all *P. fumosoroseus* colonies were sporulating which helps in differentiating this fungus from *Beauveria bassiana* and *Penicillium* sp.
Conclusions :
This method can be used to recover *Paecilomyces fumosoroseus* from the soil. This method (type of medium, technique) can be applied in a 'common' microbiology lab.

The '*Galleria* bait method' for detection of entomopathogenic fungi in soil (Zimmermann , 1986)
Principle of the method :
The larvae of several insect species (*Galleria mellonella*, greater wax moth; *Tribolium destructor*, large flour beetle; *Acanthocinus aedilis*, pine bark borer) were used for the detection of fungal pathogens (*Paecilomyces fumosoroseus, Beauveria bassiana, Metarrhizium anisopliae*) in soil.
Soil samples were sieved and put in small plastic boxes. Depending of the size of the larvae and the amount of soil, 5, 10 or 15 larvae of the bait insects are added to the moistened soil samples. The boxes were stored at room temperature and inspections were made after 1 week and again after 2 weeks. Diseased and mummified larvae were recovered and if external fungus growth was still lacking they were surface sterilized with 1% sodium hypochlorite for 3 minutes and rinsed with sterile water for some further minutes. All specimens with signs and symptoms of disease were placed in moist chambers for outgrowth of fungus.
Conclusions :
This method is practical in exploring the natural occurrence and spatial distribution of broad spectrum fungi.
Method of isolation of the fungus. No quantification of the amount of fungus can be made.
This method is well known to recover entomogenous pathogens from the soil.

B.4.2 Analytical methods - preparation (Annex IIIB 5)

B.4.2.1 Analytical methods for the analysis of the preparation (Annex IIIB 5.1)

PFR-97^{TM} 20% WDG Product Chemistry (Eyal, 1994)

The Standard Operating Procedures used in the manufacturing of the formulation can be applied in a microbiology lab in order to check the quality of the product.

928-PFR-DR	Dryer Preparation and Operation
928-PFR-QC-RM	Raw Material Quality
928-PFR-QC-CO	Assay for Contamination
928-PFR-QC-AC	Assay for Activation and Viability
928-PFR-QC-CV	Assay for Determining Conidia Viability and Germination
928-PFR-QC-CFU	Assay for CFU/g Determination
928-PFR-QC-MC	Assay for Moisture Content Determination
928-PFR-QC-BI	Bioassay for Pathogenicity Determination
928-PFR-PL/ME	Preparation of Plates and Media Necessary for QC of PFR-97
928-PFR-QC-QC	Quality Control Batch Sheet

Conclusions :
These tests allow to control the quality of the product (quality of the coformulants, CFU determination, viability , absence of contamination)

A bioassay for determining pathogenicity of entomogenous fungi on whiteflies (Landa et al., 1994)
Principle of the method :
Test procedure :
The laboratory bioassay procedure involved placing similar drops (approximately 0.003 ml per drop) of conidial suspension containing 1.0 x 10^7 conidia/ml on a sterile microscope slide using an inoculating loop. Previously collected whitefly nymphs were placed in the middle of the conidial drop (1 nymph per drop) and a total of 25 nymphs were placed on one slide. Five drops were left without nymphs to serve as a control. After the slides dried in a laminar flow hood, each slide was placed in a plastic petri dish (100 x 15 mm). The petri dishes were then incubated for 7 days at 25° C under constant light. Each fungus was assayed by using 10 petri dishes (total 250 nymphs/fungus tested).
Development of nymphs to adult :
The influence of the bioassay on development of nymphs to adult eclosion was evaluated. Early fourth instar nymphs of silver leaf whitefly *Bemisia argentifolii* and greenhouse whitefly *Trialeurodes vaporariorum* were removed from leaves and placed into drops of 0.05% Tween 80 only. The glass slides with nymphs were placed into wet chambers and kept in a growth chamber (25° C, photoperiod 16/8 h). Two hundred nymphs of both whitefly species were tested (10 wet chambers each with 20 nymphs/slide). Nymphs were individually assessed and the number of emerged adults was recorded daily for a period of 7 days.
Assessment of fungal development :
A rating system was used to assess the degree of fungal development on the whitefly nymphs. Samples were rated either daily for 1 week or at 24, 72, and 120 h. Each nymph was assessed individually under a microscope and the stade of fungus development on the nymph was recorded according to the rating index (Fungus Growth Development Index, FGDI)
Results :
Several tests were made to evaluate the effects of temperature, humidity, nutrients, surfactants, insecticides on FGDI. Several species of entomogenous fungi were compared.
No validation of the test was performed.
Conclusions :
This bioassay is useful in determining the effects of environmental factors (temperature and humidity) and additives (surfactants and adjuvants of the preparation, susceptibility to other pesticides) on the development of entomogenous fungi. It is useful as a screening tool for determining pathogenicity of new fungal strains.

This test is used to test the viability of the fungus in the formulation (quality control)

B.4.2.2 Methods to determine and quantify residues (Annex IIIB 5.2)

Methods to determine and quantify residue were not required due to the absence of residue (fungus and/or toxins) on the fruits (cucumbers and tomatoes). This issue is further discussed under point B.6 - Residues on treated products

ANNEX B

Paecilomyces fumosoroseus Strain Apopka 97

B.5 Toxicity, pathogenicity and infectivity

B.5.1 Toxicity, pathogenicity and infectivity studies - micro-organism (Annex IIB 5)

Paecilomyces fumosoroseus is a naturally occurring fungus in most countries of the world. Like most entomopathogenic fungi *P. fumosoroseus* may be found in various soil types at very low densities.
Therefore, *Paecilomyces fumosoroseus* Apopka strain 97 poses virtually no risk as an opportunistic pathogen and should not be considered as a pathogenic fungus to mammals or vertebrates due to the fact that the fungus does not grow or develop at temperatures above 35°C.
The 'technical' a.s. and the formulation do not contain any harmful mycotoxin .

B.5.1.1 Step I - Basic studies (Annex IIB 5.1)

The tests were performed with an inoculum from the exponential phase of production where maximal metabolic activity is recorded. The tests were generally made with conidia which are more stable than the blastospores.
(Total number of blastospores sprayed in the glasshouse is between 1.10^{12} and $3\ 10^{12}$ CFU/ha)

B.5.1.1.1 Acute toxicity, pathogenicity and infectivity (Annex IIB 5.1.1)

The purpose of these studies was to assess the toxicity, infectivity and pathogenicity of *Paecilomyces fumosoroseus* Apopka strain 97.

Definition of infectivity:
Invasion by and multiplication of an organism within the host tissue.

Control of viability : viability of the test fungus was verified by transferring 36 of the prills onto a yeast mannitol agar plate and incubating at 25°C for 5 days. The prills were then examined for the presence of fungal mycelium and conidia spores .

B.5.1.1.1.1 Acute oral toxicity, pathogenicity and infectivity (Annex IIB 5.1.1.1)

- Rat, single oral administration , conidia spores suspension, 1.7 x 10^6 CFU/animal (Jones, 1994)

Findings:
Mortality: all animals survived until the scheduled sacrifice.
Clinical signs: no abnormal clinical observations were noted.
Body weight: no statistically significant differences ($p<0.05$)in body weight or body weight changes were observed.
Necropsy : no abnormal findings.
Infectivity : no fungal contamination was detected in brain, mesenteric lymph nodes, blood, kidney, spleen, liver, lungs and caecum after day 1, 3, 8, 15 or 22. No faecal contamination with the fungus was noted at any time of observation period day 2, 3).

Conclusion: no signs of infectivity or pathogenicity were observed . This organism is considered to be non-toxic and non-pathogenic after a single oral dose to the rat.

Guidelines :
Experimental protocol partly in compliance with test method B.1, annex V, dir 79/831 /EEC.
The test is a limit test and only 1 dose was used.The use of one dose was justified by the notifier and accepted by the RMS :" the test was performed with a very high dose and considering the specific nature of the active substance and the inability of the fungus to survive at high temperatures, the notifier assumes that it is not necessary to conduct any further acute oral toxicity test with different doses".
The study is accepted.
GLP status:
yes (no attest of competent authority)
Material and method:
15 Sprague-Dawley (HSD:SD)rats /sex received by gavage a single oral dose of 1 ml of the test solution containing $1.7x10^6$ CFU/ml *Paecilomyces fumosoroseus* var. Apopka (lot number PFR-20B). 4 Animals/sex served as untreated shelf and room

control group. A conidia spore suspension was prepared and used to formulate the dosing solution.
3 Animals/sex were sacrificed on day 1, 3, 8, 15 and 22.

Dosage formulation: 36 prills were rehydrated with sterile water for 30 min, placed aseptically in wells and incubated at 25°C during 24 h. Distilled water was added and the plates were incubated for an additional six days. The conidia spores were harvested by adding 200µl sterile 0.05% Tween 80 to each well. Conidia spores were counted and suspensions were diluted in sterile water in order to achieve the target concentration of 10^8 spores/ml.

Viability test: was verified by transferring 36 of the prills onto a yeast mannitol agar plate and incubated at 25°C for 5 days. After the incubation period, the prills were examined for the presence of fungal mycelium and conidia spores which would indicate viability. The percent of prills which were viable was 100%.
The haemocytometer method used to count does not allow differentiation between viable and non-viable spores.
In order to determine viability, a germ tube viability assay was performed. A viable conidia spore was defined as one in which a germ tube was present and vice-versa for a non-viable conidia.The average viability (88%) of a dosing solution was determined.

True colony count: on day 1 (day of dose), an aliquot of the dosing solution was diluted in phospate buffered saline (PBS) and plated in triplicate on yeast mannitol agar plates and incubated for 7 days at 25°C. Using the CFU method, the CFU/ml of the dosing solution was found to be 1.7 x 10^6 CFU/ml.

Recovery: tissue, blood, and caecum content samples were taken from a control animal, known quantities of test fungus were added , and the homogenates were plated. Plates were incubated at 25°C for approximately 72 h to determine the presence of test fungus.The recovery in control tissues shows that the organism can be recovered with reasonable efficiency from the respective tissues.

Fecal fungal contamination: 3 animals/sex of the treated groups and 1 animal/sex from the control group were placed in metabolism cages immediately after dosing and faecal samples were collected at 24 and 48 h after dosing. On test day 4, 8, 15 and 22 caecum contents were collected at necropsy. All faecal samples and caecum content were plated and evaluated for enumeration of the test fungus.

B.5.1.1.1.2 Acute inhalation toxicity, pathogenicity and infectivity (Annex IIB 5.1.1.2)

- Rat, single intratracheal instillation, 10^6 conidia spores/animal (Jones, 1993a)

Findings:
No mortality occurred during the 72-hour observation period in the preliminary study.
Infectivity study:
Mortality: all animals survived until scheduled sacrifice.
Clinical signs: no abnormalities were noted.
Body weight: recorded on day 1, 8, 15 and 22 were normal.
Body weight gain: significant decrease for both sexes on day 8 (29% for males and 40% for females). This effect disappeared on day 15 and was not considered to be biologically significant.
Gross necropsy : 2 females exhibited discoloration or foci in the lungs.
Evaluation of infectivity:
Examination of plates from day 1 lung homogenates revealed conidia spores too numerous to count and a 1/10 dilution resulted in a spore count as presented in table B.5.1.1.1.2-1. After 4 days, lungs were free of the test fungus .Evaluation of lung tissue from animals sacrificed on days 8, 15 and 22 revealed the absence of the test fungus in all samples, therefore, the remaining 10 treated animals /sex were sacrificed on day 29 and discarded without necropsy. The caecum samples from 4 animals sacrificed on day 4 were found to contain minimal numbers of test fungus. The majority of the caecum samples from animals sacrificed on day 8, 15, and 22 were contamined by non-test fungi, however, all of the caecum samples were free of any test fungus. While there was sporadic contamination by non-test fungi, all other tissue samples from the selected organs were free of the test fungus on day 4, 8, 15, and 22. The exact quantity of tissue plated is unknown.

Table B.5.1.1.1.2-1 Colony counts of conidiospores in selected tissues

Tissue	Day 1(X ± SD)	Day 4	Day 8	Day 15	Day 22
Lung homogenate*	_ : 82 ± 24; 77 ± 24 _ : 68 ± 9 ; 76 ± 19	_ : 0 _ : 1 colony in 1 plate of 1_	0	0	0
Caecum	no data	_ : 0.18 ± 0.4; 0.90 ± 1.4 _: 0 ; 0	0	0	0
Brain	no data	0	0	0	0
Blood	no data	0	0	0	0
Kidney	no data	0	0	0	0
Spleen	no data	0	0	0	0
Liver	no data	0	0	0	0
Mesenteric lymph	no data	0	0	0	0

*: 1/10 dilution of lung homogenate on day 1 - Due to the absence of organ weight information the colony counts reflect the presence/absence of spores in a particular organ.

Clearance: of the test fungus was attained in the lungs of all treated animals by day 8 and remained absent for the remainder of the study.

Conclusion:

All animals survived both the preliminary and the infectivity studies.

Intratracheal instillation of 10^6 conidia spores per animal had no toxic effects, produced no infectivity and total clearance of the test fungus was attained by day 8.

Guidelines :

experimental protocol in compliance with test method B.2, annex V, dir 92/69 /EEC.

The test is a limit test and only 1 dosis was used. The study is accepted.

GLP status :

yes (no attest of competent authority).

Material and methods:

Preliminary test:

3 Sprague-Dawley rats/sex/dose received an intratracheal dose of the test fungus at 10^7, 10^8 and 10^9 CFU/animal after being anesthetized.

Infectivity test:

60 Sprague-Dawley rats/sex received a single intratracheal doseof 0.04 ml containing 10^6 conidia spores.

In order to establish a baseline for the evaluation of clearance of the test fungus in the lungs, 10 animals per sex from the treated groups were sacrificed within one hour of dosing and the lungs excised and homogenized. A 100 µl aliquot of each lung homogenate was plated in duplicate for numerical evaluation of conidia spores. Blood samples were drawn, and brain, kidney, liver, lungs, mesenteric lymph nodes, spleen caecum content and any gross lesion were homogenized and serially diluted in PBS to achieve concentrations of 30-300 conidia spores/ml. A 100 µl aliquot of the dilutions was plated in duplicate on YM agar, incubated at 25°C for 3-6 days, and the colony counts recorded.

Dosage formulation: 36 prills were rehydrated with sterile water for 30 min, placed aseptically in wells and incubated at 25°C during 24 h. Distilled water was added and the plates were incubated for an additional six days. The conidia spores were harvested by adding 200µl sterilewater to each well. Conidia spores were counted and suspensions were diluted in PBS in order to achieve the target concentrations

Viability and quantitation assays:

Viability was tested as in the oral toxicity test.

Concentration of the test fungus*:* on day 1 (day of dose), an aliquot of the dosing solution was diluted in phospate buffered saline (PBS) and plated in duplicate on yeast mannitol agar plates and incubated for 7 days at 25°C(preliminary study) or 72 h (infectivity study). The incubation time for the infectivity was shortened to 72 h because colonies were visible at that time. Using the CFU method, the CFU/ml of the dosing solution were found to be 4.7 x 10^5 CFU/ml, 4.5x 10^6 CFU/ml and 3.6 x 10^6 CFU/ml for the dosing solutions in the preliminary study . The CFU/ml of the dosing solution used in the infectivity study was

found to be 1.5 x 10^8 CFU/ml. These colony counts were lower than the spore count. This may be due to the nature of the CFU test which counts colonies and not single spores.

B.5.1.1.1.3 Intraperitoneal single dose (Annex IIB 5.1.1.3)

- Rat, single intraperitoneal dose , conidia spores suspension, 1.6 x 10^7 CFU/ml/animal (Jones, 1993b)

Findings:
Mortality: all animals survived until the scheduled sacrifice.
Body weight: no statistically significant differences in body weight or body weight changes were observed over the 29 day test period.
Clinical signs: no abnormal clinical signs were noted.
Necropsy : no abnormal findings.
Infectivity: no spores were detected in blood samples of any treated animal at any of the sacrifice time points (day 2, 8, 15, 22, 29). No spores were recorded from blood on day 2 of the study.

Conclusion:
The results indicate that intraperitoneal injection of 10^7 conidia spores per animal had no toxic effect, produced no infectivity, and no spores of the test fungus were detected in blood on day 2.

Guidelines:
No experimental protocol described in EEC or OECD.
The study is accepted.
GLP :
yes (no attest of competent authority)
Material and methods:
18 Sprague-Dawley (HSD:SD) rats/sex received a single intraperitoneal dose of 1 ml with a concentration of 1.6 x 10^7 CFU/ml of *Paecilomyces fumosoroseus* var. Apopka (chem.article T07444).
7 Animals/sex served as control.

Dosage formulation: 36 prills were rehydrated with sterile water for 30 min, placed aseptically in wells and incubated at 25°C during 24 h. Distilled water was added and the plates were incubated for an additional six days. The conidia spores were harvested by adding 200 µl sterile 0.05% tween 80 to each well. Conidia spores were counted .
For the infectivity study, the conidia spore suspensions were diluted in sterile water in order to achieve a minimum concentration of 10^7 spores/ml.
Viability and quantitation assays:
Viability was tested as in the oral toxicity test.
The concentration of the test fungus was performed. A conidia spore suspension was prepared and used to formulate the dosing solution. The conidia spore suspension was diluted and the spore count was determined using a hemacytometer. The average viability (88%) was determined by germination . Also on day 1 (day of dose), the dosing solution was diluted in PBS and plated in triplicate on YM agar plate and incubated for 6 days at 24°C. Using the CFU method, the CFU/ml of the dosing solution was found to be 1.6 x 10^7 CFU/ml.

Recovery and enumeration:
Recovery of the plating procedure was tested by "spiking" control blood samples with test fungus. Blood was diluted (1:1) with the spore suspension and 100 µl were placed in a hemacytometer to determine the presence of conidia spores and mycelium. The blood samples were then plated in duplicate and cultured for 72 h. Based on the CFU count in an equal volume of spore suspension diluted with PBS, recovery from blood was calculated to be 11.6%.

B.5.1.1.1.4 Skin irritation (Annex IIB 5.1.1.4)

-Rabbits, 4 hour dermal exposure, 0.5 g undiluted blastospores and mycelium (Wenk, 1994a)

Findings:
Score erythema (24+48+72h) = 0
Score edema (24 +48+ 72h) = 0
A very slight erythema (score = 1) was observed on the treated flank of 3/6 rabbits after one hour reading. At twenty four hours reading, this effect was only detected in one rabbit. Forty-eight hours after exposure, no dermal irritation was observed in any animal.

Conclusion:
Paecilomyces fumosoroseus has no irritant properties under the conditions of the assay.

Guidelines:
Study protocol in compliance with method B.4, dir 79/831/EEC.
The study is accepted.
GLP :
yes (no attest of competent authority)
Material and methods:
6 Male New Zealand white rabbits were exposed to 0.5 g *Paecilomyces fumosoroseus* (lot number 7/13/93A), undiluted (blastospores and mycelium), moistened with deionized water, and covered by gauze pads and adhesive tape for 4 hours.

Dosage formulation:
CFU's were verified by plating on YM agar and incubated at 26-30°C for 1-5 days. The CFU count was determined : 2.3 x 10^8 CFU/g.

B.5.1.1.1.5 Eye irritation (Annex IIB 5.1.1.5)

- 6 Rabbits, blastospores and mycelium, 0.1 ml containing 2.5-5.4 x 10^7 CFU (Wenk, 1994b)

Findings:
In the pretest screen, only slight irritations were observed by 24 hours .
Score conjunctiva:
Discharge 24h/48h/72h = 0.277
Chemosis 24h/48h/72h = 0.055
Redness 24h/48h/72h = 0.222
Score cornea opacity:
without fluoresceine: 24h/48h/72h = 0
with fluoresceine : 24h/48h/72h = 0.222
Score iris 24h/48h/72h = 0
The test fungus produced initially slight irritation in all 6 rabbits which reversed completely by 4 days following the treatment.
Conclusion:
Paecilomyces fumosoroseus is not irritating to the eye.

Guidelines:
Experimental protocol in compliance with test method B.5, Dir 79/831/EEC.
The study is accepted.
GLP status:
yes (no attest of competent authority)
Material and methods:
0.1 ml of semi solid lot of *Paecilomyces fumosoroseus* (lot PFR 10-31-94; pH: 5.35 was used for the pretest screen and PFR 11/1/94 ; pH 5.28 was used for the complete test) was instilled into the left eye of 6 male New Zealand albino rabbits. Eyes were examined 1 hour, 24, 48, 72 hours, 4 and 7 days after treatment.
The study was divided into 2 phases. The first was a Pretest screen in which 1 animal was initially tested. In the complete test, 5 animals were used . The complete test started 24 hours after the pretest.
Both eyes were evaluated 1 hour and 24 hours following treatment and fluorescein was instilled in order to verify the possible

occurrence of corneal opacity or ulceration.
Dosage formulation: CFUs were verified by plating on Yeast-Malt Agar and incubating at 25°-30°C for 2 days. Actual dose preparations contained 5.4 x 10^8 CFU/ml(pretest) and 2.5 x 10^8 CFU/ml (complete test). Therefore about 2.5-5.4 x 10^7 CFU were administered to each animal.

B.5.1.1.1.6 Skin sensitization (Annex IIB 5.1.1.6)

- Guinea pigs, Buehler test, blastospores and mycelium, 0.4 ml of 25% (2.6 x 10^7 CFU/g), and 0.4 g of 50% (4.6 x 10^7 CFU/g), 75% (2.2 x 10^8 CFU/g) w/v, and the neat chemical (Wenk, 1994c)

Findings (range-finding test):
No reactions were observed at any of the four concentrations and the neat fungus was chosen for the sensitization test
4 Guinea-pigs received a single 0.4 ml dose of 25% and 0.4 g of 50% , and 75% and the neat chemical, each at a different site. The compound was applied for 6 hours. Exposed areas were scored for irritation after 24 and 48 h. The highest dose, which resulted in no to very faint erythema, was chosen for the test.
Test procedure:
2 groups of 10 animals and 2 groups of 5 animals were used. To 1 group of 10 animals, the test fungus (neat) was applied to the left flank for each induction dose, once weekly for 3 weeks; challenge dose was applied to the right flank 2 weeks later. The same protocol was applied to a group of 5 animals exposed to the positive control. The other 10 and 5 animals were exposed once, at time of challenge dose.

Findings (main test):
No reactions were observed at any of the 4 concentrations in the range-finding study and the neat fungus was chosen for the sensitization test.
Body weights were not adversely affected by the test fungus.
After the first induction, no erythema was observed in any test animals 24 hours after the first , second or third induction. No test-fungus-treated animals exhibited skin reactions from exposure to the test fungus after challenge.

Conclusion:
Paecilomyces fumosoroseus Apopka strain 97 is not a dermal sensitizer in the test conditions..

Guidelines:
Experimental protocol not fully in compliance wtih test method B.6, Dir 79/831/EEC.
Deviation from ofiicial protocol: 10 animals instead of 20 in the treated group and 5 in control instead of 10.
The study is accepted.
GLP :
yes (no attest of competent authority)
Material and methods:
The Buehler test was used.
Paecilomyces fumosoroseus var Apopka (lot n°.7/13/93A) was administered dermally to Hartley guinea pigs, once weekly for three weeks for the induction phase. Animals were challenged after a two week rest period. The CFUs for the bulk material was 1.6 x 10^8 CFU/g.
Dosage formulations:
Concentrations of 0.4 ml of 25% (2.6 x 10^7 CFU/g),and 0.4 g of 50% (4.6 x 10^7 CFU/g), 75% (2.2 x 10^8 CFU/g) w/v, and the neat chemical were used for the sensitization test. Dinitrochlorobenzene was used as positive control (0.05% in 90% ethanol).

B.5.1.1.2 Genotoxicity testing (Annex IIB 5.1.2)

B.5.1.1.2.1 *In vitro* studies (Annex IIB 5.1.2.1)

- *Salmonella*/mammalian microsome reverse mutation assay, sonicated blastospores and mycelium : 100, 333, 667, 1000, 3330 or 5000 µg/plate, with/without S9 mix. (Ames test) (Lawlor, 1994)

Findings:
Negative results were noted. Slight test article particulate was noted at 333 µg/plate and higher in both the presence and absence of S9 mix.

Conclusion:
Paecilomyces fumosoroseus Apopka strain 97 did not cause a positive increase in the numbers of histidine revertants per plate with or without S9 mix.

Guidelines:
Experimental protocol in compliance with method B.14, dir 92/69/EEC.
GLP :
Yes (no attest of competent authority)
Material and methods:
A dose-range finding assay was performed using tester strain TA100 with or without S9.
The mutagenicity test was realized with tester strains TA 98, 100, 1535, 1537 and TA1538 using the plate incorporation assay.
Light brown granules of *Paecilomyces fumosoroseus* Apopka strain 97 were homogeneously suspended in DMSO and sonicated for 3 hours. The most concentrated suspension was at 50 mg/ml and used for the subsequent dilutions.
In a preliminar study, 10 doses from 5000 to 6.67 µg/plate were tested. No cytotoxicity was observed up to 5000 µg/plate with or without S9 mix (Aroclor pretreated rat liver).
The mutagenicity test was performed in two individual experiments. Doses were 100, 333, 667, 1000, 3330 or 5000 µg/plate, with/without S9 mix. Each experimental point was in triplicate.
The study was realized in good experimental conditions: appropriate controls gave the expected responses.

No other studies were conducted to detect any possible mutagenic effect of *Paecilomyces fumosoroseus* Apopka strain 97. The notifier is not aware of any of these types of adverse effects arising from the use of *Paecilomyces fumosoroseus* strain Apopka 97 or any other strains of the fungus.
The strain does not grow at 37°C. Moreover, the active substance is a pure product, without contaminations and is completely free of all known mycotoxins.

B.5.1.1.2.2 *In vivo* studies in somatic cells (Annex IIB 5.1.2.2)

Based on the results reported in the other toxicity studies as well as the negative results reported in the Ames test, the RMS may anticipate that *Paecilomyces fumosoroseus* strain Apopka 97 does not possess any mutagenic properties in any other *in vitro* or *in vivo* genotoxicity test.

B.5.1.1.3 Cell culture studies (viruses or viroids) (Annex IIB 5.1.3)

This item does not apply to fungi.

B.5.1.1.4 Short-term toxicity, pathogenicity and infectivity (Annex IIB 5.1.4)

In the acute toxicity studies, using different routes of exposure, *Paecilomyces fumosoroseus* Apopka strain 97 was generally not recovered in the biological fluids and when it was present, no particular secondary effects were noted in exposed animals. It is therefore, not possible to determine target organs, NOAELs or any dose-effect relationship . The concentrations at which the tests were performed were comparable to the concentrations that should be sprayed in practical conditions.

In view of the limited lifespan of the fungus *Paecilomyces fumosoroseus* strain Apopka 97 at human body temperatures and the absence of symptoms of infectivity, pathogenicity or toxicity in the acute studies, it is not necessary to perform short-term toxicity studies. A toxic response after repeated administration can be expected only on the assumption that the organism produces a toxin.This is not the case for this strain ; moreover, no reports in open literature mention infections consecutive to this specific strain. It is an endemic and generally occurring fungus. It is not possible and not necessary to determine a NOAEL.

B.5.1.2 Step II - Additional studies (Annex IIB 5.2)

B.5.1.2.1 Acute percutaneous toxicity, pathogenicity and infectivity (Annex IIB 5.2.1)

-Rabbits , dermal application, blastospores and mycelium, 2 g (8.0 x 10^8 CFU/g), 24 h (Wenk, 1994d)

Findings:
Mortality: all animals survived the fourteen days observation period.
Clinical signs:
- local signs: erythema at the application site was commonly observed in all animals tested starting day 2, over a period of 5 study days. Symptoms gradually disappeared and were totally reversed by day 8. At first observation, grades of erythema ranged from mild to severe.
- No systemic effects were observed.
Body weight: increase : 14% in males and 14.6% in females
Necropsy: no gross lesions were noted.
Conclusion:
Transient primary dermal irritation was noted at the exposure site of all animals in the form of mild to severe erythema which reversed within four days.

Guidelines:
experimental protocol not fully in compliance with method B.1, dir 79/831.
This is a limit test. The high dose used in the test has some irritating properties.
The study is accepted.
GLP status:
yes
Material and methods:
5 New Zealand albino rabbits/sex were exposed to 2 g (8.0 x 10^8 CFU/g) *Paecilomyces fumosoroseus* var. Apopka (lot n°. PFR 33A) administered undiluted and moistened with water to the clipped skin under adhesive tape during 24 hours.

Dosage formulation: CFU were verified ; an aliquot of the bulk material was serially diluted in PBS and plated in triplicate on yeast malt agar. The plates were incubated for 3 days at 25°-30°C. Thse concentration of the test organism in the dose preparation was 8.0x10^8 CFU/g.

B.5.1.2.2 Genotoxicity - In vivo studies in germ cells (Annex IIB 5.2.2)

Because of the negative findings in the genotoxicity test mentioned in B.5.1.1.2, *in vivo* tests have not been performed.

B.5.1.2.3 Short-term toxicity : other routes (inhalation and intraperitoneal/intravenous) (Annex IIB 5.2.3)

See point B.5.1.1.4. The RMS considers this step not necessary.

B.5.1.3 Step III - Specific toxicity, pathogenicity and infectivity studies under immunosuppression (Annex IIB 5.3)

Paecilomyces fumosoroseus strain Apopka 97 does not contain toxins ; previously reported studies indicate that the fungus may not cause long-term adverse effects. Therefore, the RMS considers that step III is not necessary.

B.5.1.4 Medical data (Annex IIB 5.4)

B.5.1.4.1 Medical surveillance on manufacturing plant personnel (Annex IIB 5.4.1)

Reports of occupational health surveillance programmes, supported with detailed information on the design of the programme and on exposure to the micro-organism, are not included in the dossier.

Informations concerning employees participating in research activities involving *Paecilomyces fumosoroseus* strain Apopka 97 are included. No reports of toxicity, hypersensitivity, infectivity or other adverse effects are mentioned.
- Research on *Paecilomyces fumosoroseus* was performed in Poland since 1994 where 10 people were exposed daily to conidia, blastospores and mycelial fragments which were sprayed on plants in different commercial greenhouses and laboratories. No symptoms of illness, infectivity or any adverses effects were reported.
- Researchers in USA involved in the development of the commercial product handled fungal preparations containing conidia, blastospores and mycelial fragments and sprayed it out on plants in the greenhouse and laboratory. From time to time, certain employees have had minor cuts and scrapes, became pregnant, had minor surgery and one had severe burns from an alcohol lamp. Nobody became ill, was infected or showed any adverse symptoms from their daily exposure to *Paecilomyces fumosoroseus* strain Apopka 97 . (Mass et al., -)

B.5.1.4.2 Sensitization/allergenicity observations, if appropriate (Annex IIB 5.4.2)

Although most insect pathogenic fungi are considered to be harmless to man, allergic reactions have been reported after harvesting of spores of fungi. It was suggested that this reaction resulted from inhaling spores. Some reports suggest that no harmful effects were observed after inhalation of blastospores of fungi such as *B. bassiana* and long sleeves and gloves halted the response, whereas the use of a respirator alone did not (Roberts and Yendol, -)
Proper attire and careful formulation should reduce the hazard to operators.

The fungus is formulated as a water dispersible granulate , a dustfree formulation which therefore poses no risk for inhalation during preparation of the spray solution. *Paecilomyces fumosoroseus* strain Apopka 97 will be applied with large quantities of water where few possibilities exist for the inhalation of spray solution during application. Blastospores are known to be little allergenic.

B.5.1.4.3 Direct observation, e.g. clinical cases (Annex IIB 5.4.3)

From open literature, no infectious-allergic diseases were reported following exposure to *Paecilomyces fumosoroseus.*

Primary or secondary infection of the lungs with fungi of the *Paecilomyces* genus (*P.variotii* and *P.viridis*) were reported and give rise to the development of infectious allergic bronchopulmonary paecilomycosis, characterized by the presence of chronic allergic interstitial pneumonia and obstructive bronchitis, bronchial asthma, total and pulmonary eosinophilia, presence of tissue parasitic form of the fungus in sputum, blood, pulmonary tissue, presence of allergen-specific IgE and /or IgG antibodies in patients' sera, immediate or double (20 min and 6 h)

reaction of the skin to administration of allergen of *Paecilomyces*, by not infrequent combination of lung damage and impairment of other organs, as well as by chronic relapses (Akhunova, 1992).

B.5.1.4.4 Observations on exposure of the general population and epidemiological data, if appropriate (Annex IIB 5.4.4)

see point B.5.1.4.1

B.5.1.1.5 Proposed treatment : first aid measures, antidotes, medical treatment (Annex IIB 5.4.5)

It is not a human pathogen. Therefore, no treatment is proposed.

B.5.1.5 Summary of mammalian toxicity, pathogenicity and infectivity and overall evaluation (Annex IIB 5.5)

Acute toxicity:
Tests conducted with *P.fumosoroseus* strain Apopka 97 indicate that this fungus is not toxic and is devoid of infectivity, toxicity or pathogenicity after acute oral, dermal, inhalation or intraperitoneal administration. It is not a skin irritant. Eye instillation did not cause any toxic effect. Acute toxicity tests have not led to any mortality in test animals in any single test.
No fungal contamination was observed in brain, mesenteric lymph nodes, blood, kidneys, spleen, liver, lungs and caecum , 1 day or more after oral exposure. No faecal contamination was noted at any time of the observation period (day 2, 3).
After intraperitoneal administration, no conidia were detected in blood samples at day 2, 8, 15, 22 or 29.
After a single intratracheal instillation, lungs were free of test fungus after 8 days. At this time, caecum of some animals contain minimal numbers of the test fungus.
In order to evaluate the sensitization ability of *Paecilomyces ,* the Buehler test was used. Repeated dermal application of the compound is more relevant than intradermal administration, and permits to anticipate that no sensitization should occur after repeated dermal exposure.

According to the EU draft, the micro-organism in the form in which it will be used, has to be tested in acute toxicity tests. However, the notifier used for oral, intratracheal, intraperitoneal routes the conidia form which is more stable than the blastospores and seems then more adapted to those routes

Dermal toxicity, skin and eye irritation and skin sensitization were performed with blastospores and mycelium which are present in the formulation.

Table B.5.1.5-1: Summary of acute toxicity of *Paecilomyces fumosoroseus* strain Apopka 97

Test	**Dosage formulation**	**Dose**	**Findings**	**References**
Oral, rat	Conidia spores suspension	1.7 x 10^6 CFU/animal	negative	Jones , 1994
Intratracheal, rat	Conidia spores suspension	10^6 conidia spores/animal	discoloration or foci in lungs of 2 _	Jones , 1993a
Dermal, rabbit	blastospores and mycelium	2 g/animal (8 x 10^8 CFU/g)	transient irritation completely reversible after 4 d	Wenk, 1994d
Intraperitonal, rat	Conidia spores suspension	1.7 x 10^6 CFU/ml/animal	no infectivity, no toxicity	Jones , 1993b
Skin irritation	blastospores and mycelium	10^8 CFU/g (0.5 g/animal)	negative	Wenk, 1994a
Eye irritation	blastospores and mycelium	2.5-5.4 x 10^7 CFU / animal.(0.1 ml)	negative	Wenk, 1994b
skin sensitization Buehler test	blastospores and mycelium	induction: at 10^7; 3.10^7; $6.3.10^9$ CFU/g challenge: 10^6CFU/g	negative	Wenk, 1994c

Genotoxicity:
A sonicated suspension of *Paecilomyces fumosoroseus* was unable to induce point mutations in *Salmonella typhimurium* in the Ames test.

Short-term toxicity :
In view of the limited lifespan of the fungus *Paecilomyces fumosoroseus* strain Apopka 97 at human body temperatures and the absence of symptoms of infectivity, pathogenicity or toxicity in the acute studies, it is not necessary to perform short-term toxicity studies. A toxic response after repeated administration can be expected only on the assumption that the organism produce a toxin.This is not the case for this strain ; moreover, no reports in open literature mention infections consecutive to this specific strain. It is an endemic and generally occurring fungus. It is not possible and not necessary to determine a NAOEL.

Immunosuppresive effects:
Micro-organisms which normally cause infection in humans with suppressed immunosystem are well known ; such effects were not reported for *Paecilomyces fumosoroseus* Apopka 97.

Observations on human exposure:
Reports of occupational health surveillance programmes, supported with detailed information on the design of the programme and on exposure to the micro-organism are not included in the dossier.
Informations concerning employees participating in research activities involving *Paecilomyces fumosoroseus* are included. No toxicity, hypersensitivity, infectivity or other adverse effects were observed .
Research on *Paecilomyces fumosoroseus* was performed in Poland since 1994 and 10 people were exposed daily to conidia, blastospores and mycelial fragments which were sprayed on plants in different commercial greenhouses and laboratories. No symptoms of illness, infectivity or any adverses effects were reported.
Researchers in USA handled fungal preparations containing conidia, blastospores and mycelial fragments and sprayed it out on plants in the greenhouse and laboratory. From time to time, certain employees have had minors cuts and scrapes, become pregnant, had minor surgery and one had severe burns from an alcohol lamp. Never had anyone become ill, infected or shown any adverse symptoms from their daily exposure to *Paecilomyces fumosoroseus* Apopka 97.

Allergenicity :
Although most insect pathogenic fungi are considered to be harmless to man, allergic reactions resulting from inhaling spores have been reported after harvesting of spores of fungi. Some reports suggest that no harmful effects were observed after inhalation of blastospores of fungi such as *B. bassiana* and long sleeves and gloves halted the response, whereas the use of a respirator alone did not (Roberts and Yendol, -)
Paecilomyces fumosoroseus is formulated as a water dispersible granulate , a dustfree formulation which therefore poses no risk for inhalation during preparation of the spray solution.

From open literature, no infectious-allergic diseases were reported following exposure to *Paecilomyces fumosoroseus*.

Primary or secondary infection provoked by related species:
Primary or secondary infection of the lungs with fungi of the *Paecilomyces* family (*P.variotii and P.viridis*) were reported and give rise to the development of infectious allergic bronchopulmonary paecilomycosis (Akhunova, 1991).

Acceptable daily intake (ADI)

Paecilomyces fumosoroseus is a naturally occurring fungus geographically widespread and a common entomopathogenic of insect pests. It does not grow at temperature above 32°C; it is not a human pathogen. In the acute toxicity studies, no effects related to exposure on the fungus were reported.
Therefore, no ADI is proposed.

Acceptable Operator Exposure level (AOEL)

In the acute toxicity studies, using different routes of exposure, *Paecilomyces fumosoroseus* strain Apopka 97 was generally not recovered in the biological fluids and when it was present, no particular secondary effects were noted in exposed animals. It is therefore, not possible to determine target organs, NOAELs or any dose-effect relationship . The concentrations at which the toxicity tests were performed were comparable to the concentrations that should be sprayed in practical conditions.
Paecilomyces fumosoroseus is unable to grow at temperatures above 32°C.
It is not possible and not necessary to determine a NOAEL.

Drinking water limit.

No ADI was proposed, therefore it is not necessary to determine a drinking water limit.

B.5.2 Toxicity, pathogenicity and infectivity studies - formulation PREFERAL (Annex IIIB 7)

The active substance in PREFERAL WG is *Paecilomyces fumosoroseus* Apopka strain 97. The formulation contains mycelia and blastospores of *Paecilomyces fumosoroseus* Apopka strain 97 at a concentration of 2 10^9 CFU/g. All inert ingredients used in the formulation are approved for food use.

B.5.2.1 Step I - Basic acute toxicity studies (Annex IIIB 7.1)

PREFERAL WG is merely a granular formulation containing the active substance and food grade inert ingredients that are all listed by the FDA as generally recognized as safe. Therefore, nothing in those inert ingredients should reasonably be able to cause any adverse effects.
For these reasons, the RMS accepts that acute toxicity studies with the formulation should be waived.
Moreover, tests have been performed with the a.s., the only reactive substance in the preparation.

B.5.2.1.1 Acute oral toxicity (Annex IIIB 7.1.1)

See point B.5.1.1.1.1
An acute oral toxicity study was conducted with a combination of conidia and mycelia of *Paecilomyces fumosoroseus* Apopka strain 97. This test showed no signs of toxicity, pathogenicity, or infectivity in any of the test animals.

B.5.2.1.2 Acute inhalation toxicity (Annex IIIB 7.1.2)

See point B.5.1.1.1.2
An acute pulmonary toxicity/pathogenicity study was conducted with conidia of *Paecilomyces fumosoroseus* Apopka strain 97. This test showed no signs of toxicity, pathogenicity, or infectivity in any of the test animals.

B.5.2.1.3 Acute percutaneous toxicity (Annex IIIB 7.1.3)

See point B.5.1.2.1
An acute dermal toxicity study was conducted with *Paecilomyces fumosoroseus* Apopka strain 97. This test showed no signs of toxicity at 10^9 CFU/animal.

B.5.2.2 Step II - Additional acute toxicity studies (Annex IIIB 7.2)

B.5.2.2.1 Skin irritation (Annex IIIB 7.2.1)

See point B.5.1.1.1.4
A primary dermal irritation study was conducted with *Paecilomyces fumosoroseus* Apopka strain 97. This test showed that the test product is a slight irritant, the effects of which are reversible within 72 h at 10^8 CFU/animal.

B.5.2.2.2 Eye irritation (Annex IIIB 7.2.2)

See point B.5.1.1.1.5
A primary eye irritation study was conducted with *Paecilomyces fumosoroseus* Apopka strain 97. This test showed that the test article is non irritating at $\geq 10^7$ CFU/animal.

B.5.2.2.3 Skin sensitization (Annex IIIB 7.2.3)

See point B.5.1.1.1.6
An dermal sensitization study was conducted with *Paecilomyces fumosoroseus* Apopka strain 97. This test showed that the strain is not a sensitizer at 10^7 CFU/animal .

B.5.2.3 Data on exposure (Annex IIIB 7.3)

Paecilomyces fumosoroseus will only be applied in limited cultivations under cover and on limited surfaces .
PREFERAL WG (water dispersible granule) will be used to support the biological control in tunnels and greenhouses. The application rate is 2.10^{12} - 6.10^{12} CFU/ha corresponding to 1-3 kg formulation/ha. The number of applications will be made dependent on the extent of parasitism by *Encarsia formosa* and the degree of infestation. Spraying may be used as application method.

- No toxic effects are expected to occur after exposure to *Paecilomyces fumosoroseus* : the fungus is not a pathogen and does not produce secondary metabolites. It is not a skin sensitizer.
- The fungus is formulated as a water dispersible granulate , a dustfree formulation which therefore poses no risk for inhalation during preparation of the spray solution. *Paecilomyces fumosoroseus* strain Apopka 97 will be applied with large quantities of water where few possibilities exist for the inhalation of spray solution during application. Blastospores are known to be little allergenic.
- In greenhouses, continuously important numbers of fungus spores are present and insect pathogenic fungi occur naturally . The continuous exposure of growers to fungus spores would theoretically lead to allergy for fungus spores amongst growers.
- During the past few years, several tests have been carried out with *Paecilomyces fumosoroseus* strain Apopka 97, and people were therefore exposed to this strain. No single negative effect was observed. *Paecilomyces fumosoroseus* strain Apopka 97 is a generally occurring fungus in the nature, so that there is a constant exposure.
- Regularly large epizootics of *P.fumosoroseus* take place amongst whiteflies populations in humid climates such as in Florida, Pakistan, India,.... From these areas where high concentrations of *P.fumosoroseus* spores occur, no cases of infectivity, pathogenicity, toxicity or allergies are reported to be caused by this fungus. The absence of effects reported by the notifier was supported by the absence of pathogenicity reported in the literature, after thorough examination by the RMS.

No special precautions such as resting times or safety times are required.
Special clothes and shoes are recommended. Contact with skin, eyes of clothes must be avoided.
The applicator should wear a mask while spraying this fungus.

B.5.2.4 Available toxicological data relating to non-active substances (Annex IIIB 7.4)

No data, not necessary.

B.5.2.5 Supplementary studies for combinations of preparations (Annex IIIB 7.5)

No data, not necessary.

ANNEX B

Paecilomyces fumosoroseus Strain Apopka 97

B.6 Residues in or on treated products, food and feed

B.6.1 Persistence and likelihood of multiplication of the active substance in or on crops or feedingstuffs or foodstuffs (Annex IIB 6.1)

The following justification was submitted by the notifier in order to assess the low persistence of the a.s. on crops and foodstuffs and to waive the requirement of residue data on edible crops. These statments were accepted as valid by the RMS.

"The basic starting point is that before *Paecilomyces fumosoroseus* strain Apopka 97 reaches the consumer, after being used as a biological insecticide, this fungus must pass through several "sieves" which results in a continuous decrease of the number of spores on the fruits.

1) *Paecilomyces fumosoroseus* strain Apopka 97 will be used within an integrated pest management strategy of whiteflies in greenhouses as a selective correction product, in other words based on the observations ("monitoring"). *Paecilomyces fumosoroseus* strain Apopka 97 will only be applied in spot treatments for the adjustment of the biological control of whiteflies. The biological control of whiteflies is done by parasitoids (*Encarsia formosa*) and predators (*Macrolophus caliginosus*).

2) From the total number of blastospores sprayed, (between 2.10^{12} - 6.10^{12} CFU/ha), only a minimal fraction will end up on the fruits.

3) Both tomatoes and cucumbers have a water-repellent fruitskin that contains a lot of different waxes (hydrofobic). The waxes prevent the hydrophilic spores which are dispersed in water from attaching to the fruits.

4) Both conidia- and blastospores are not survival forms.

5) The lack of a substrate (insects) on the fruits, on which the fungus could grow, makes that the fungus spores can not develop any further.

6) Since the fruits can not transpire, there is no boundary layer of high relative air humidity around the fruits and therefore spores of PFR can't germinate even if spores are present on the fruit. The fungus will be effective on insects that are present on underside of the leaf because a boundary layer of high relative humidity will be there due to evapotranspiration.

7) All further treatments between harvesting and consumption (harvesting, sorting out, packing, storing in cooled conditions, washing, ...) of the fruits will destroy or remove the possible remaining spores."

B.6.2 Further information required (Annex IIB 6.2)

B.6.2.1 Non-viable residues (Annex IIB 6.2.1)

Not required due to the absence of toxin production (See point B.1.1.4.2)

B.6.2.2 Viable residues (Annex IIB 6.2.2)

Not required. The fungus is not infective nor pathogenic to mammals.

B.6.3 Summary and evaluation of residue behaviour (Annex IIB 6.3)

Paecilomyces fumosoroseus strain Apopka 97 is a naturally occurring fungus geographically widespread and a common entomopathogenic of insect pests.

No ADI was proposed for this active substance due to the following reasons :
- The absence of growth at temperature above 32°C.
- The absence of effects related to exposure to the fungus in the toxicity studies as well as the observations of persons which were in contact with the fungus revealed the absence of human infectivity, pathogenicity and toxicity. No dose-related levels can be fixed.
- The presence of toxins in the formulation is excluded due to the fermentation process conditions which are applied.
- The fungus is not present on edible crops and foodstuff (cucumber and tomato).

The informations which were provided as well as the absence of ADI definition clearly demonstrate that the risk for the consumer resulting from the presence of residue (fungus, possible metabolites) on/in treated product and food is negligible.
The establishment of a residue definition, of MRLs and the calculation of the potential exposure of the consumer are therefore meaningless. Consequently, residue field trials, analytical methods to quantify residue, re-entry periods and PHI are not required.

ANNEX B

Paecilomyces fumosoroseus Strain Apopka 97

B.7 Fate and behaviour in the environment

B.7 Fate and behaviour in the environment (Annex IIB 7)

B.7.1 Persistence and multiplication (Annex IIB 7.1)

B.7.1.1 Persistence and multiplication in soil (Annex IIB 7.1.1)

The persistence and multiplication in soil of the specific strain *Paecilomyces fumosoroseus* strain Apopka 97 was evaluated by Hunsberger (1993). Other references from the open literature confirm the results of this study.
The persistence and stability of *Paecilomyces fumosoroseus* in practical conditions is very low. *Paecilomyces fumosoroseus* Apopka strain 97 should not present any environmental persistence problems in soil or water. As a natural product, *Paecilomyces fumosoroseus* Apopka strain 97 is routinely found in the environment.

A selective medium for the detection of *Paecilomyces fumosoroseus* in greenhouse soil and its potential as a microbial control agent of *Diaprepes abbreviatus* larvae in containerized citrus (Hunsberger, 1993)
Material and methods :
Viability and infectivity of *Paecilomyces fumosoroseus* strain Apopka 97 blastospores which were mixed to soil was investigated.
Viability in the soil:
0.5, 1 and 3 g prills were incorporated into 18 g of dried unsterilized greenhouse soil (50% coarse Canadian peat moss, 40% perlite, 10% vermiculite, pH : 6.5-7.5; total porosity : 70%) by placing the soil into a waxed paper container and adding the prill treatment. For each prill concentration, four soil moistures (0, 30, 60 and 100% saturation) were tested for conidiation in time. The containers were sealed with parafilm to prevent moisture loss and then placed in a greenhouse (soil temperatures ranged from 24-28°C (mean = 26°C); containers were shaded with brown paper cover). 18 replicates/treatment.
3 soil samples/treatment were collected at day 0, 3, 6, 12, 24 and 38 post treatment
Soil samples were placed in centrifuge tube containing 30 ml sterilized water, sonicated and vortexed in order to dislodge conidia. Serial dilutions of the stock suspensions were placed on selective medium (see B.4.1.2.1) to determine the concentration in the soil expressed in CFU.
Bioassay :
At day 3 , 6 and 12 5 g soil sample/container were removed and placed into bioassay units to determine the infectivity to *Diaprepes abbreviatus* larvae (development of sporulating *P. fumosoroseus* on the larvae)

Findings :
Table B.7.1.1-1 : Estimated conidial population densities (CFU/cc soil) and percent mycosis of *D. abbreviatus* larvae (in brackets) in greenhouse soil at 26 ± 2 °C

Soil moisture (%)	**Concentration rate (g blastospores /18 g soil)**	**Days post treatment**					
		0	**3**	**6**	**12**	**24**	**38**
0	0	0	0 (0)	0 (0)	0 (0)	0	0
	0.5	0	0 (0)	0 (0)	0 (0)	0	0
	1.0	0	0 (0)	0 (0)	0 (4.8)	0	0
	3.0	0	0 (0)	0 (0)	0 (0)	0	0
30	0	0	0 (0)	0 (0)	0 (0)	0	0
	0.5	0	0 (0)	1.4×10^4 (1.1)	1.0×10^5 (1.8)	2.6×10^4	0
	1.0	0	0 (0)	8.0×10^4 (0)	3.2×10^4 (8.8)	1.1×10^4	0
	3.0	0	0 (0)	2.1×10^4 (0)	3.2×10^4 (22.2)	3.5×10^4	0
60	0	0	0 (0)	0 (0)	0 (0)	0	0
	0.5	0	0 (0)	8.3×10^4 (0)	2.5×10^5 (0)	2.5×10^4	0
	1.0	0	0 (0)	3.4×10^4 (0)	5.9×10^4 (0)	8.6×10^4	0
	3.0	0	0 (0)	2.1×10^4 (0)	6.6×10^4 (0)	2.1×10^4	0

Soil moisture (%)	Concentration rate (g blastospores /18 g soil)	Days post treatment					
		0	**3**	**6**	**12**	**24**	**38**
100	0 0.5 1.0 3.0	0 0 0 0	0 (0) 0 (0) 0 (1.5) 0 (1.8)	0 (0) 1.1×10^4 (0) 1.3×10^4 (11.1) 2.1×10^4 (0)	0 (0) 1.1×10^5 (0) 2.9×10^4 (0) 5.6×10^4 (5.4)	0 1.6×10^4 - 2.1×10^4	0 0 0 0

Conclusions :
At 0% soil moisture, no production of conidia occurs.
The production of conidia was not related to the different levels of soil moisture and of the initial amounts of blastospores added to the soil. At day 38 no viable conidia were detected by colony counts.
Mycosis was not related to initial amounts of blastospores added to the soil. Mycosis was not dependent from the moisture level of the soil.

Soil contains fungi and bacteria which produce substances with antibiotic properties. Thus the growth of *Paecilomyces fumosoroseus* (Wize) Brown and Smith, for instance is slowed down by metabolites of soil microorganisms such as *Penicillium citrinum, Penicillium rubrum* and *Aspergillus* species (Eyal, 1994d).

The persistence of conidia in soil was investigated with an experimental biodegradation method using a trap technique at 19°C. At various times of incubation, traps were collected to study changes in biomass and inoculum potential. The infection potential of *Paecilomyces fumosoroseus* was substantially degraded after 6 months incubation (70-80% dry weight loss) (Fargues and Robert, 1985).

B.7.1.2 Persistence and multiplication in water (Annex IIB 7.1.2)

Information on the persistence and multiplication in water is not required : application of the formulation in greenhouse excludes contamination of surface waters.

One study on the survivorship of spores was provided.
Percent survivorship of *Paecilomyces fumosoroseus* relative to elevated temperature (40°C), sonication, and UV irradiation is 0 second, 10 minutes , and 120 seconds respectively
Property of blastospore of *Paecilomyces fumosoroseus* was compared against that of its conidia. Blastospore was less stable than conidia when it was stored under dried state and stored in water. In the susceptibility tests with various antiseptics (mercuric chloride, phenol, cresol soap solution), no significant difference was seen between blastospore and conidia. Resistance of blastospores to UV treatment and high temperature treatment was nearly identical with conidia. (Shimizu, 1988).

B.7.1.3 Persistence and multiplication in air (Annex IIB 7.1.3)

Information of the persistence in air is not required : toxicological data showed that the fungus is not a human pathogen. There is no risk for the operator/worker/bystander.

B.7.2 Mobility (Annex IIB 7.2)

It is expected that the mobility of the *Paecilomyces fumosoroseus* strain Apopka 97 and its possible spread to the environment will be limited since the only intended uses are in greenhouses. The possible spread of the this micro-organism strain would not be hazardous since this species is a naturally occurring fungus geographically widespread, which is found on a wide variety of insect hosts and substrates.
No study is required since no toxicity, infectivity nor pathogenicity have been reported.

ANNEX B

Paecilomyces fumosoroseus Strain Apopka 97

B.8 Ecotoxicology

B.8.1 Effects on birds (Annex IIB 8.1, Annex IIIB 10.1)

Paecilomyces fumosoroseus Apopka 97: An avian oral pathogenicity and toxicity study in the northern bobwhite (Frey, et al., 1994)
Guideline:
FIFRA guidelines subdivision M 154A-16
GLP:
yes
Material and Methods:
Test substance: PFR-97^{TM} 20 % WDG (WG containing 2.10^{9} CFU/g *Paecilomyces fumosoroseus* Apopka strain 97)
Test species: *Colinus virginianus*
Number of organisms:
3 x 10 birds for treatment with *Paecilomyces fumosoroseus*
2 x 10 birds for attenuated control and for negative control
Applied concentrations:
- Negative control,
- attenuated control (autoclaved formulation),
- treatment : 2500 mg formulation/kg bw/d

Exposure route:
Fifty northern bobwhite quails, undifferentiated by sex were individually weighed and administered the appropriate dosing by gavage . *Paecilomyces fumosoroseus* Apopka Strain 97 was administered to a total of 30 birds in 6 of the pens. The resultant total dosage was approximately 2.5 x 10^{9} CFU/kg of body weight over the 5-day dosing period. The control groups consisted of a negative control and attenuated control. Each of the control groups consisted of ten birds in two pens. The nominal dosage for the attenuated control was identical to the treatment group except that the test substance was inactivated. Birds in the negative control group were administered deionized water at a dose of 1.0 % (v/w) of body weight for five days in order to subject the control birds to the same stresses from handling and dosing as birds in treatment groups.
Test conditions:
temp.: 25.8 °C ± 1.0 °C
humidity 69 % ± 10 %
Findings:
Mortality : no treatment related mortality
Body weight : no effect
Clinical signs : no evidence of pathogenicity or treatment related effects in the treatment or attenuated control group.
Conclusions:
The study is acceptable. The NOED of *Paecilomyces fumosoroseus* Apopka Strain 97 administered to northern bobwhite *Colinus virginianus* was 2.5 x 10^{9} CFU/kg bw/d for five days.

No other study is required.
The risk of *Paecilomyces fumosoroseus* to birds is negligible (absence of any effect in the toxicity test, no exposure of the birds by the application in glasshouse)

B.8.2 Effects on aquatic organisms (Annex IIB 8.2, Annex IIIB 10.2)

B.8.2.1 Acute toxicity and/or pathogenicity and infectivity to fish (Annex IIB 8.2.1)

Not required. Water organisms are unlikely to be exposed to the fungus after its application as insecticide in glasshouse. .

B.8.2.2 Acute toxicity to aquatic invertebrates (Annex IIB 8.2.2)

Not required. Water organisms are unlikely to be exposed to the fungus after its application as insecticide in glasshouse.

B.8.2.3 Effects on algal growth (Annex IIB 8.2.3)

Not required. Water organisms are unlikely to be exposed to the fungus after its application as insecticide in glasshouse.

B.8.3 Effects on bees (Annex IIB 8.3, Annex IIIB 10.3)

Several studies were performed on bumblebees (*Bombus terrestris*) which is a species intensively used in glasshouse as pollinator. Honeybees are not exposed to *Paecilomyces* (Honeybees are not used as pollinator in glasshouse and are not attracted by tomato or cucumber flowers)

Toxicity determination with respect to bumblebees of the product PFR (*Paecilomyces fumosoroseus*) (De Wael, et al., 1994)
Guidelines :
BBA Teil VI 23-1 Auswirkungen von Pflanzenschutzmitteln auf die Honigbiene.
GLP :
No
Material and methods :
Test substance : Formulation PREFERAL (WG containing 200 g/kg *Paecilomyces fumosoroseus*)
Test species : Bumblebees (*Bombus terrestris*) from an artificially reared colony
Number of organisms : 10 bumblebees X 2 replicates per treatment
Types of test : lab test (72 hours)
Applied concentrations : 1 and 3 g PREFERAL/ l solution, in water or sucrose solution depending on the route of exposure, parathion as positive standard.
Test conditions : 29°C, relative humidity = 60%
Exposure routes : individual feeding, ad libitum feeding, contact, direct spraying, inhalation
Findings :
Mortality after 72 hours
Oral administration (individual) : 5%
Oral administration (ad libitum) : 0%
Contact toxicity : 0%
Direct spraying : 0%
Inhalation toxicity : 0%
Conclusions :
No effects of the fungus were observed during the first 3 days of the experiment. Nevertheless the duration of the test is relatively short in comparison with the infective cycle of the test fungus and does not allow to fully assess the possible effects after a longer period of incubation. More information is required to determine the toxicity of the fungus to bumblebees.

Toxicity of the entomopathogenic fungus *Paecilomyces fumosoroseus* strain Apopka 97 (PreFeRal WG) for bumblebee brood (*Bombus terrestris* L.) (Bolckmans, 1994)
Guidelines :
Test protocol developed by De Wael L. et al; (1994)
GLP :
No
Material and methods :
Test substance : Formulation PREFERAL (WG containing 200 g/kg *Paecilomyces fumosoroseus*)
Test species : Bumblebees (*Bombus terrestris*)
Number of organisms : 5 young bumblee colonies with 10-20 adults and with all life stages present/ treatment
Types of test : lab test (± 30 days)
Applied concentrations : 3 g PREFERAL/ l sugar solution; 0.15 g teflubenzuron /l sugar solution; sugar solution as negative control
Test conditions : 29°C, relative humidity = 60%
Exposure routes : the colonies were fed exactly 24 h with the solutions to be tested. Afterwards the colonies were fed with fresh sugar solution.
Findings :
'During ± 1 month the dead larvae were counted in each treatment (20 counts)'
The cumulative counts at the end of the period were
negative control : 34 dead larvae
PREFERAL : 40 dead larvae
positive control : 299 dead larvae
The raw data were reported in a letter of the notifier sent to the RMS. The validity of these data cannot be assessed.
Conclusions :
This test showed that PREFERAL is harmless to bumblebee brood.

A third study was dedicated to the determination of the effects of *Paecilomyces fumosoroseus* strain Apopka 97 sprayed onto the bumblebee brood itself. No effect on the brood was observed. Due to the lack of the raw data and a clear definition of the protocol this test will not be further assessed.

B.8.4 Effects on non-target arthropods other than bees (Annex IIB 8.4, Annex IIIB 10.4)

Toxicity of Preferal (*Paecilomyces fumosoroseus* strain Apopka 97) to the predatory mite *Phytoseiulus persimilis*; semi-field; Ekotox rapport nr.2 (Sterk, 1994a)
Guideline:
IOBC and EPPO test methods for semi-field trials
GLP:
No
Material and Methods:
Test substance:
PREFERAL (*Paecilomyces fumosoroseus* Strain Apopka 97) (WG containing 2.10^9 CFU/g)
Test species:
Phytoseiulus persimilis A.H., predatory mite, adult females
Number of organisms:
10 adult females x 4 replicates per treatment
Type of test:
semi-field
Applied concentrations:
100 g PREFERAL/ 100 l water, 2.10^9 CFU/l till run-off
fenpropathrin (toxic) and fenbutatinoxid (selective) were used as standard.
Exposure route:
4 beanplants (Limburgse vroege) per object were placed in the compartment, infested with living mobile stages of two spotted spider mite, *Tetranychus urticae*, as prey. After the treatment ten *Phytoseiulus persimilis* adult females

of the same age were carefully placed with a small brush on the one remaining leaf of each test plant.
A drilled round plate with a ring of glue at the rim was placed around the bottom of each plant. This enables to distinguish between direct toxicity and eventual repellent effects. The number of surviving adults and the numbers of eggs and larvae on the leaves, and the dead or sticked larvae in the plate, were counted after 10 days.
Test conditions:
Temperature and humidity in the glasshouse were recorded during the whole trial but not reported.
Findings:
Table B.8.4-1: Effects of PREFERAL on *Phytoseiulus persimilis* in a semi-field trial

Evaluation criteria	Control	Treatment	Endpoints
Number of living larvae and adult on 4 x 1 leaf after 10 days	40	41	Abbott: E= -2.50 %

Conclusions:
Preferal (*Paecilomyces fumosoroseus* strain Apopka 97), at the used dose rate of 2.10^9 CFU/l, has no toxicity at all on adult females of the predatory mite *Phytoseiulus persimilis*. Also, fertility and fecundity seem to be not effected. No repellent effect was observed on the plates. PREFERAL is non-toxic (class 1) to *Phytoseiulus persimilis* in semi-field trials.

Toxicity of Preferal (*Paecilomyces fumosoroseus* strain Apopka 97) to the predatory mite *Amblyseius degenerans*, Trial 1; semi-field; Ekotox rapport nr.3 (Sterk, 1994b)
Guideline:
IOBC and EPPO test methods for semi-field trials on *Phytoseiulus persimilis* and adapted for *Amblyseius degenerans*.
GLP:
No
Material and Methods:
Test substance:
PREFERAL (*Paecilomyces fumosoroseus* strain Apopka 97) (WG containing 2.10^9 CFU/g)
Test species:
Amblyseius degenerans, predatory mite, adult
Number of organisms:
20 adult mites x 3 replicates per treatment
Type of test:
semi-field
Applied concentrations:
100 g PREFERAL/ 100 l water, 2.10^9 CFU/l till run-off. Tebufenpyrad (toxic), avermectin (toxic) and endosulfan (selective) were used as standard.
Exposure route:
3 ricinus plants per object were placed in the compartment, with pollen as food. After the treatment ten adult mites of the same age were carefully placed with a small brush on each of the two remaining leaves of each test plant.
A drilled round plate with a ring of glue at the rim was placed around the bottom of each plant. This enables to distinguish between direct toxicity and eventual repellent effects.
The number of surviving adults and the numbers of eggs and larvae on the leaves, and the dead or sticked larvae in the plate, were counted after 10 days. No information on the eggs counts.
Test conditions:
Temperature and humidity in the glasshouse were recorded during the whole trial but not reported.
Findings:
Table B.8.4-2 : Effects of PREFERAL on *Amblyseius degenerans* in a semi-field trial

Evaluation criteria	Control	Treatment	Endpoints
Number of living larvae and adults on 3 x 2 leaves after 10 days	44	33	Abbott: E = 25.00 %

Conclusions:
Preferal (*Paecilomyces fumosoroseus* strain Apopka 97), at the used dose rate of 2.10^9 CFU/l, has a very limited effect on adults of the predatory mite, *Amblyseius degenerans*. Preferal is non-toxic (class 1) to *Amblyseius degenerans* in semi-field trials. No repellent effect of the compound was observed on the plates.

Toxicity of Preferal (*Paecilomyces fumosoroseus* strain Apopka 97) to the predatory mite *Amblyseius degenerans*, Trial 2; semi-field; Ekotox rapport nr.4 (Sterk, 1994d)
Guideline:
IOBC and EPPO test methods for semi-field trials on *Phytoseiulus persimilis* and adapted for *Amblyseius degenerans*.
GLP:
No
Material and Methods:
Test substance:
PREFERAL (*Paecilomyces fumosoroseus* strain Apopka 97) (WG containing 2.10^9 CFU/g)
Test species:
Amblyseius degenerans, predatory mite, adult
Number of organisms:
20 adult mites x 3 replicates per treatment
Type of test:
semi-field
Applied concentrations:
100 g PREFERAL/ 100 l water, 2.10^9 CFU/l till run-off
Tebufenpyrad (toxic), avermectin (toxic) and endosulfan (selective) were used as standard.
Exposure route:
3 bean plants (Limburgse vroege) per object were placed in the compartment with ricinus pollen as food. After the treatment ten adult mites of the same age were carefully placed with a small brush on the one remaining leaf of each test plant.
A drilled round plate with a ring of glue at the rim was placed around the bottom of each plant. This enables to distinguish between direct toxicity and eventual repellent effects.
The number of surviving adults and the numbers of eggs and larvae on the leaves, and the dead or sticked larvae in the plate, were counted after 10 days. No information on the eggs counts was provided.
Test conditions:
Temperature and humidity in the glasshouse were recorded during the whole trial but not reported.
Findings:
Table B.8.4-3: Effects of PREFERAL on *Amblyseius degenerans* in a semi-field trial

Evaluation criteria	Control	Treatment	Endpoints
Number of living larvae and adults on 3 x1 leaves after 10 days	25	31	Abbott: E = -24.00 %

Conclusions:
Preferal (*Paecilomyces fumosoroseus* strain Apopka 97), at the used dose rate of 2.10^9 CFU/l, has no toxic effect on adults of the predatory mite, *Amblyseius degenerans*. Preferal is non-toxic (class I) to *Amblyseius degenerans* in semi-field trials. No repellent effect of the compound was observed on the plates.

Toxicity of Preferal (*Paecilomyces fumosoroseus* strain Apopka 97) to the predatory mite *Amblyseius degenerans*, Trial 3; semi-field; Ekotox rapport nr.5 (Sterk, 1994e)

Guideline:

IOBC and EPPO test methods for semi-field trials on *Phytoseiulus persimilis* and adapted for *Amblyseius degenerans*.

GLP:

No

Material and Methods:

Test substance:

PREFERAL (*Paecilomyces fumosoroseus* strain Apopka 97) (WG containing 2.10^9 CFU/g)

Test species:

Amblyseius degenerans, predatory adult mite

Number of organisms:

10 adult mites x 5 replicates

Type of test:

semi-field

Applied concentrations:

100 g PREFERAL/ 100 l water, 2.10^9 CFU/l till run-off

Pyriproxifen (selective), endosulfan (selective), and fenpropathrin (toxic) were used as standard.

Exposure route:

5 ricinus plants per object were placed in the compartment with ricinus pollen as food. After the treatment ten adult mites of the same age were carefully placed with a small brush on the one remaining leaf of each test plant.

A drilled round plate with a ring of glue at the rim was placed around the bottom of each plant. This enables to distinguish between direct toxicity and eventual repellent effects.

The number of surviving adults and the numbers of eggs and larvae on the leaves, and the dead or sticked larvae in the plate, were counted after 15 days. No information on the eggs counts was provided.

Test conditions:

Temperature and humidity in the glasshouse were recorded during the whole trial but not reported.

Findings:

Table 8.4- 4: Effects of PREFERAL on *Amblyseius degenerans* in a semi-field trial

Evaluation criteria	Control	Treatment	Endpoints
Number of living larvae and adults on 5 x1 leaves after 15 days	30	36	Abbott:E = -20.00 %

Conclusions:

Preferal (*Paecilomyces fumosoroseus* strain Apopka 97), at the used dose rate of 2.10^9 CFU/l, has no toxic effect on adults of the predatory mite, *Amblyseius degenerans*. Preferal is non-toxic (class I) in semi-field trials. No repellent effetc of the compound was observed on the plates.

Toxicity of Preferal (*Paecilomyces fumosoroseus* strain Apopka 97) to the flower bug *Orius insidiosus;* semi-field; Ekotox rapport nr.6 (Sterk,1994e)

Guideline:

non-published IOBC test methods for semi-field trials on *Orius insidiosus*

GLP:

No

Material and Methods:

Test substance:

PREFERAL (*Paecilomyces fumosoroseus* strain Apopka 97) (WG containing 2.10^9 CFU/g)

Test species:

Orius insidiosus, predatory bug (L1 - L2 larvae)

Number of organisms:

10 L1/L2 larvae x 5 replicates per treatment

Type of test:
semi-field
Applied concentrations:
100 g PREFERAL/ 100 l water, 2.10^9 CFU/ l till run-off
Fenpropathrin (toxic) and pyriproxifen (selective) were used as standard.
Exposure route:
5 young aubergines were covered with pollen and eggs of *Ephestia* as prey. After this five L1/L2 larvae of ± the same age were carefully placed with a small brush on each of the two fully grown leaves of each test plant.
A drilled round plate with a ring of glue at the rim was placed around the bottom of each plant. This enables to distinguish between direct toxicity and eventual repellent effects. The number of surviving larvae on the leaves, and the dead or sticked larvae on the plate, were counted after 8 days.
Test conditions:
Temperature and humidity in the glasshouse were recorded during the whole trial but not reported.
Findings:
Table B.8.4-5: Effects of PREFERAL on *Orius insidiosus* in a semi-field trial

Evaluation criteria	Control	Treatment	Endpoints
Number of living larvae and adults on 5 x 2 leaves after 8 days	46	41	Abbott: E = 10.87 %

Conclusions:
PREFERAL (*Paecilomyces fumosoroseus* strain Apopka 97), at the used dose rate of 2.10^9 CFU/l, has no toxicity at all on young larvae of the flower bug *Orius insidiosus*. Preferal is non-toxic (class I) to *Orius insidiosus* in semi-field trials.

Toxicity of Preferal (*Paecilomyces fumosoroseus* strain Apopka 97) to the flower bug *Orius insidiosus*; semi-field; Ekotox rapport nr.7 (Sterk, 1994f)
Guideline:
non-published IOBC test methods for semi-field trials on *Orius insidiosus*
GLP:
No
Material and Methods:
Test substance:
PREFERAL (*Paecilomyces fumosoroseus* strain Apopka 97) (WG containing 2.10^9 CFU/g)
Test species:
Orius insidiosus, flower bug, L1-L2 larvae
Number of organisms:
40 L1/L2 larvae x 4 replicates per treatment
Type of test:
semi-field
Applied concentrations:
300 g PREFERAL/ 100 l water, 6.10^9 CFU/l till run-off
Fenpropathrin (toxic) and tebufenpyrad (toxic) were used as standard.
Exposure route:
4 x 4 young beanplants (Limburgse Vroege) of ± 25 cm height were covered with pollen and eggs of *Sitotroga* as prey. After this, five L1/L2 larvae of ± the same age were carefully placed with a small brush on each of the two fully grown leaves of each test plant.
The plants were placed in an isomo plate, with a ring of glue. This enables to distinguish between direct toxicity and eventual repellent effects.
The number of surviving larvae on the leaves, and the dead or sticked larvae on the plate, were counted after 6 days.
Test conditions:

Temperature and humidity in the glasshouse were recorded during the whole trial but not reported.
Findings:
Table B.8.4-6 : Effects of PREFERAL on *Orius insidiosus* in a semi-field trial

Evaluation criteria	Control	Treatment	Endpoints
Number of living larvae on 4 x 4 plants after 6 days	44	51	Abbott: E = - 15.91 %

Conclusions:
PREFERAL (*Paecilomyces fumosoroseus* strain Apopka 97), at the used dose rate of 6.10^9 CFU/l formulated compound, has no toxicity at all on young larvae of the flower bug *Orius insidiosus*. Preferal is non-toxic (class I) to *Orius insidiosus* in semi-field trials. No repellent effects were observed.

Toxicity of Preferal (*Paecilomyces fumosoroseus* strain Apopka 97) to the flower bug *Orius insidiosus*; semi-field; Ekotox rapport nr.8 (Sterk, 1994g)
Guideline:
non-published IOBC test methods for semi-field trials on *Orius insidiosus*
GLP:
No
Material and Methods:
Test substance:
PREFERAL (*Paecilomyces fumosoroseus* strain Apopka 97) (WG containing 2.10^9 CFU/g)
Test species:
Orius insidiosus, flower bug, L1-L2 larvae
Number of organisms:
40 L1/L2 larvae x 3 replicates per treatment
Type of test:
semi-field
Applied concentrations:
300 g PREFERAL/ 100 l water, 6.10^9 CFU/l till run-off.
Buprofezine (selective), fenpropathrin (toxic) and pyridaben (toxic) were used as standard.
Exposure route:
3 x 4 young beanplants (Limburgse Vroege) of ± 25 cm height were covered with pollen and eggs of *Sitotroga* as prey. After this five L1/L2 larvae of ± the same age were carefully placed with a small brush on each of the two fully grown leaves of each test plant.
The plants were placed in an isomo plate, with a ring of glue. This enables to distinguish between direct toxicity and eventual repellent effects.
The number of surviving larvae on the leaves, and the dead or sticked larvae on the plate, were counted after 10 days.
Test conditions:
Temperature and humidity in the glasshouse were recorded during the whole trial but not reported.
Findings:
Table B.8.4-7 : Effects of PREFERAL on *Orius insidiosus* in a semi-field trial

Evaluation criteria	Control	Treatment	Endpoints
Number of living larvae on 3 x 4 plants after 10 days	42	46	Abbott: E = - 9.52 %

Conclusions:
PREFERAL (*Paecilomyces fumosoroseus* strain Apopka 97), at the used dose rate of 6.10^9 CFU/l formulated compound, has no toxicity at all on young larvae of the flower bug *Orius insidiosus*. Preferal is non-toxic (class I) to

Orius insidiosus in semi-field trials. No repellent effects were observed.

Toxicity of Preferal (*Paecilomyces fumosoroseus* strain Apopka 97) to the flower bug *Orius laevigatus*; semi-field; Ekotox rapport nr.9 (Sterk, 1994h)
Guideline:
non-published IOBC test methods for semi-field trials on *Orius laevigatus*
GLP:
No
Material and Methods:
Test substance:
PREFERAL (*Paecilomyces fumosoroseus* strain Apopka 97) (WG containing 2.10^9 CFU/g)
Test species:
Orius laevigatus, flower bug, L1/L2 larvae
Number of organisms:
40 L1/L2 larvae x 3 replicates per treatment
Type of test:
semi-field
Applied concentrations:
300 g PREFERAL/ 100 l water, 6.10^9 CFU/l till run-off.
Fenpropathrin (toxic), tebufenpyrad (toxic) and pyriproxifen (selective) were used as standard.
Exposure route:
3 x 4 young beanplants (Limburgse Vroege) of ± 25 cm height were infested with pollen and eggs of *Sitotroga* as prey. After this, five L1/L2 larvae of ± the same age were carefully placed with a small brush on each of the two fully grown leaves of each test plant.
The plants were placed in an isomo plate, with a ring of glue. This enables to distinguish between direct toxicity and eventual repellent effects.
The number of surviving larvae on the leaves, and the dead or sticked larvae on the plate, were counted after 8 days.
Test conditions:
Temperature and humidity in the glasshouse were recorded during the whole trial but not reported.
Findings:
Table B.8.4.-8: Effects of PREFERAL on *Orius laevigatus* in a semi-field trial

Evaluation criteria	Control	Treatment	Endpoints
Number of living larvae on 3 x 4 plants after 8 days	70	73	Abbott: E = - 4.29 %

Conclusions:
PREFERAL (*Paecilomyces fumosoroseus* strain Apopka 97), at the used dose rate of 6.10^9 CFU/l formulated compound, has no toxicity at all on young larvae of the flower bug *Orius laevigatus*. Preferal is non-toxic (class I) to *Orius laevigatus* in semi-field trials. No repellent effects were observed.

Toxicity of Preferal (*Paecilomyces fumosoroseus* strain Apopka 97) to the predatory bug *Macrolophus caliginosus*; semi-field; Ekotox rapport nr.10 (Sterk, 1994i)
Guideline:
non-published IOBC test methods for semi-field trials on *Macrolophus caliginosus*
GLP:
No
Material and Methods:
Test substance:
PREFERAL (*Paecilomyces fumosoroseus* strain Apopka 97) (WG containing 2.10^9 CFU/g)
Test species:

Marcrolophus caliginosus, predatory bug, L1/L2 larvae
Number of organisms:
5 L1/L2 larvae x 8 replicates per treatment
Type of test:
semi-field
Applied concentrations:
100 g PREFERAL/ 100 l water, 2.10^9 CFU/l till run-off.
Fenpropathrin (toxic), pyriproxifen (selective), avermectin (toxic) and phosalone (selective) were used as standard.
Exposure route:
8 young tomato plants of ± 40 cm height were covered with pollen and eggs of *Ephestia* as prey. After this five L1/L2 larvae of ± the same age were carefully placed with a small brush on each of the test plants.
The number of surviving larvae on the leaves was counted after 8 days.
Test conditions:
Temperature and humidity in the glasshouse were recorded during the whole trial but not reported.
Findings:
Table B.8.4-9 : Effects of PREFERAL on *Macrolophus caliginosus* in a semi-field trial

Evaluation criteria	Control	Treatment	Endpoints
Number of living larvae on 8 x 1 plant after 8 days	29	27	Abbott: E = 6.9 %

Conclusions:
PREFERAL (*Paecilomyces fumosoroseus* strain Apopka 97), at the used dose rate of 2.10^9 CFU/l, has no toxicity at all on young larvae of the predatory bug *Macrolophus caliginosus*. Preferal is non-toxic (class I) to *Macrolophus caliginosus* in semi-field trials.

Toxicity of Preferal (*Paecilomyces fumosoroseus* strain Apopka 97) to the predatory bug *Macrolophus caliginosus*; semi-field; Ekotox rapport nr.11 (Sterk, 1994j)
Guideline:
non-published IOBC test methods for semi-field trials on *Macrolophus caliginosus*
GLP:
No
Material and Methods:
Test substance:
PREFERAL (*Paecilomyces fumosoroseus* strain Apopka 97) (WG containing 2.10^9 CFU/g)
Test species:
Macrolophus caliginosus, predatory bug, L1/L2 larvae
Number of organisms:
5 L1/L2 larvae x 5 replicates per treatment
Type of test:
semi-field
Applied concentrations:
300 g PREFERAL/ 100 l water, 6.10^9 CFU/l till run-off.
Fenpropathrin (toxic), pyriproxifen (selective), avermectin (toxic) and phosalone (selective) were used as standard.
Exposure route:
5 young beanplants (Limburgse Vroege) were cut and covered with eggs of *Sitotroga* as prey. After this, five L1/L2 larvae of ± the same age were carefully placed with a small brush on each of the test plants. The number of surviving larvae on the leaves was counted after 3, 7, 11 and 14 days.
Test conditions:
Temperature and humidity in the glasshouse were recorded during the whole trial but not reported.

Findings:
Table B.8.4-10 : Effects of PREFERAL on *Macrolophus caliginosus* in a semi-field trial

Evaluation criteria	Control	Treatment	Endpoints
Number of living larvae on 5 x 1 plant after 3 days	23	19	Abbott: E = 17.4 %
Number of living larvae on 5 x 1 plant after 7 days	24	15	Abbott: E = 37.5 %
Number of living larvae on 5 x 1 plant after 11 days	21	14	Abbott: E = 33.3 %
Number of living larvae on 5 x 1 plant after 15 days	19	14	Abbott: E = 26.3 %

Conclusions:
PREFERAL (*Paecilomyces fumosoroseus* strain Apopka 97), at the very high dose rate of 6.10^9 CFU/l formulated compound, is slightly harmful (class 2) on young larvae of the predatory bug *Macrolophus caliginosus*. PREFERAL can be used without any restriction in biological control of the white fly. The dead larvae were not observed in order to determine the cause of the death.

Laboratory assessment of the toxicity of the entomopathogenic fungus PFR to the greenhouse whitefly *Trialeurodes vaporariorum*, parasite wasp *Encarsia formosa* (Degheele et al., 1994)
Guideline:
Guidelines for the evaluation of side-effects of pesticides - *Encarsia formosa*. (Oomen, 1985)
GLP:
No
Material and Methods:
Test substance:
PREFERAL (*Paecilomyces fumosoroseus* strain Apopka 97) (WG containing 2.10^9 CFU/g)
Test species:
Encarsia formosa
Number of organisms:
15 females X 3 replicates per treatment
Type of test:
lab test
Applied concentrations:
3 g PREFERAL/ l water, 6.10^9 CFU/l. 1 mg solution/cm^2
Water control
Exposure route:
15 females are introduced into cages and supplied with honey as food. On days 2, 4 and 7 the surviving insects are counted and are offered for parasitization host scales on bean leaf for a period of 4 hours.
Test conditions:
temperature : 20-22°C
light/dark : 16h / 8h
relative humidity : 70-80%
Findings:

Table B.8.4.-11 : Effects of PREFERAL on *Encarsia formosa* in a laboratory test (mortality and parasitization)

Evaluation criteria	Control	Treatment	Endpoints
Mortality of the females at days 2, 4 and 7	0%	0%	
Mean number of scales parasitized/female at day 2	2.06	2.40	no reduction
at day 4	2.08	2.28	no reduction
at day 7	2.44	2.20	10% reduction

Conclusions:
PREFERAL (*Paecilomyces fumosoroseus* strain Apopka 97) should be classified as harmless to *Encarsia formosa*

Several studies were not taken into account in the final evaluation due to their very poor quality. Nevertheless they didn't show harmful effects.

- Susceptibility of the Convergent Lady Beetle (Coleoptera Coccinellidae) to Four Entomogenous Fungi (James and al., 1993)
- Influence of PFR on beneficial insects, *Aphidoletes sp.* (anonymous, 1992)
- PFR-SPWF and Parasites (Transvena) In Vivo-Poinsettias (anonymous, 1993)
- *Paecilomyces fumosoroseus*, Margosan-O and *Amblyseius cucumeris* in integrated pest management of Western Flower Thrips (*Frankliniella occidentalis*) (anonymous, 1993)

Table B.8.4-12 : Summary of effects of PREFERAL on non-target terrestrial arthropods

Test species	Test system	Duration of exposure	Results	Risk Assessment	References
Phytoseiulus persimilis	Semi-field test	10 days	E = -2.5 % (dose : 2.10^9 CFU/l water)	harmless	(Sterk, 1994a)
Amblyseius degenerans	Semi-field test	10 days	E = 25 % (dose : 2.10^9 CFU/l water)	harmless	(Sterk, 1994b)
Amblyseius degenerans	Semi-field test	10 days	E = -24 % (dose : 2.10^9 CFU/l water)	harmless	(Sterk, 1994c)
Amblyseius degenerans	Semi-field test	15 days	E = -20 % (dose : 2.10^9 CFU/l water)	harmless	(Sterk, 1994d)
Orius insidiosus	Semi-field test	8 days	E = 11 % (dose : 2.10^9 CFU/l water)	harmless	(Sterk, 1994e)
Orius insidiosus	Semi-field test	6 days	E = - 16 % (dose : 6.10^9 CFU/l water)	harmless	(Sterk, 1994f)
Orius insidiosus	Semi-field test	10 days	E = -9 % (dose : 6.10^9 CFU/l water)	harmless	(Sterk, 1994g)
Orius laevigatus	Semi-field test	8 days	E = -4 % (dose : 6.10^9 CFU/l water)	harmless	(Sterk, 1994h)
Macrolophus caliginosus	Semi-field test	8 days	E = 7 % (dose : 2.10^9 CFU/l water)	harmless	(Sterk, 1994i)
Macrolophus caliginosus	Semi-field test	15 days	E = 26 % (dose : 6.10^9 CFU/l water)	slightly harmful	(Sterk, 1994j)
Encarsia formosa	lab test	7 days	no effect on the mortality of the adults nor the parasitization	harmless	Degheele, et al. 1994)

B.8.5 Effects on earthworms (Annex IIB 8.5, Annex IIIB 10.5)

The study is not required. *Paecilomyces fumosoroseus* is a fungus found very commonly in soil.

B.8.6 Additional studies (Annex IIB 8.6, Annex IIIB 10.6)

Studies on other soil organisms and on soil microbial activity (respiration, soil nitrogen transformation) are not required because :

- *Paecilomyces fumosoroseus* is a fungus which is found very commonly in soil.

- The PEC evaluation made by the rapporteur showed that the impact of this fungus is negligible :
The applied dose rate of 266-532 µg/kg soil (equivalent to an application rate of 200-400 g blastospora/ha dispersed in a 5 cm soil layer of density 1.5 g/cm^3) has to be related to a 'normal' microbiotic fauna of 200 mg/kg soil. It seems therefore unexpected that the presence of this organism will disturb the microbial processes of the soil.

ANNEX B

Paecilomyces fumosoroseus Strain Apopka 97

B.9 Classification and labelling

B.9.1 Proposals including justification of the proposals for the classification and labelling of the active substance in accordance with Directive 67/548/EEC

The micro-organism is not classified according to its toxicological profile.

The directive 67/548/EEC seems not to be the most adequate to classify micro-organisms. The classification of the micro-organisms used as plant protection products should be based on the principles applied for the protection of workers from risks related to biological agents.

B.9.2 Proposals including justification of the proposals for the classification and labelling of the preparation in accordance with Directive 78/631/EEC

The formulation is not classified according to its toxicological profile. General safety precautions can be decided on Member State level.