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	_ : $3.2\ 10^4 \pm 9.5\ 10^3$ CFU/lungs _ : $2.8\ 10^4 \pm 1.1\ 10^4$ CFU/lungs	_ : 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

Clearance: Colony counts at day 1 showed that 3% of the instilled dose was recovered in the lungs at day 1. These figures indicated that clearance was important and that no invasion nor colonization of the lungs occurred. Complete clearance 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. No invasion nor colonization of the lungs was observed. 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.

Toxicity of PreFeRal (*Paecilomyces fumosoroseus* (Wize) Brown & Smith, strain Apopka 9) on adult workers and larvae of *Bombus terrestris* L.) (Bolckmans, 1998)
Guidelines :
Test protocol developed by Biobest
GLP :
No
Material and methods :
Test substance : Formulation PREFERAL (WG containing 200 g/kg *Paecilomyces fumosoroseus*)
Test species : Bumblebees (*Bombus terrestris*)
Number of organisms : 10 young female worker bumblebees were placed in a plastic hive and were allowed to produce broth during 1 week. 4 replicates per treatment were used.
Types of test : semi-field test (± 39 days)
Applied concentrations : PREFERAL at the dose rates of 0.1 and 0.2% in sugarwater. Dimethoate (toxic to adults), teflubenzuron (toxic to larvae), acetamiprid (control with effects on the behaviour of the adults) and lufenuron were used as control.
Test conditions : 29°C.
Exposure routes : the colonies were fed with pesticide solutions for 39 days. Pesticide solutions were replaced twice a week.
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.