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भारत सरकार

Government of India

कृषि एवं किसान कल्याण मंत्रालय

Ministry of Agriculture & Farmers Welfare

कृषि, सहकारिता एवं किसान कल्याण विभाग

Department of Agriculture, Co-operation & Farmers Welfare

वनस्पति संरक्षण, संगरोध एवं संग्रह निदेशालय

DIRECTORATE OF PLANT PROTECTION, QUARANTINE & STORAGE

केंद्रीय कीटनाशी बोर्ड ए वम पंजीकरण समिति

Central Insecticides Board and Registration Committee

एन. एच. 4, फरीदाबाद (हरियाणा)-121001

N.H. IV, FARIDABAD (HARYANA)-121001

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Dated the 24 May, 2021

PUBLIC NOTICE

Subject-Harmonization of existing toxicological guidelines/protocols for registration of bio-pesticides in India-regarding.

In partial modification of this Sectt's earlier public notice dated 24.03.2021 on the above mentioned subject, please find enclosed the biopesticide ecotoxicity study protocols approved by RC at agenda item No. 10.16 in 426th meeting, with the request to submit comments within 60 days period from the date of uploading of this Public Notice on the website of Dte. of PPQ&S. The reply may be sent through email to cibsecy@nic.in and socir2.ppqs-agri@gov.in.

This has the approval of APPA & Secretary (CIB&RC).

Encl.- As above.

Suman

(Suman Jakhar)
Section Officer

Copy to:

1. Pesticide Associations
2. Chairman, Registration Committee
3. PPS to JS (PP)
4. PPS to PPA
5. PPS to Secretary (CIB&RC)
6. IT Cell, HQ, Faridabad for uploading the same on Directorate's website.

Avian oral toxicity

Objective: To assess and evaluate the toxic or pathogenic characteristics of a Microbial Pesticide in avian species.

Purpose: The purpose of the avian oral toxicity is to provide initial information on the toxicity, infectivity, and/or pathogenicity of microbial pesticides. It provides information on health hazards likely to arise from exposure by the oral route in birds.

Principle/Rationale: An evaluation of acute toxicity data should include the relationship, if any, between the bird's exposure to the test substance and the incidence and severity of all abnormalities, including behavioral and clinical abnormalities, the reversibility of observed abnormalities, gross lesions, body weight changes, effects on mortality, and any other toxic effects

Species: Testing shall be performed on one avian species, one insectivorous and one herbivorous (preferably bobwhite quail). The use of two species of birds with differing diets is recommended in order to take into account possible differences in gastrointestinal physiology. Other species may be used but a justification must be supplied based on increased susceptibility to the MPCA or ecological considerations which preclude the use of recommended species.

Age: At the beginning of the test period, 14 to 24 days old birds should be used in this test. Within a given test, all birds shall be as near the same age as possible.

Weight: Weight variation should be within + 20 % of the mean ideal weight of the animal.

Housing and Feeding Conditions:
Temperature: 15^{o}-27°C
Relative Humidity: 50-60% (However, humidity should not be below 30% or exceed 70% at any given point of time).
Lighting: 8 hours light and 16 hour dark cycle
Diet and water: *Ad libitum* standard diet, (specific to the species) and filtered water (free from contamination).
Acclimatization: The animals should be acclimatized to the laboratory conditions for at least 14 days prior to dosing by keeping them in individual cages.

Groups and number of birds required:

Control group

(i) A negative, non dosed control group should be performed.
(ii) An infectivity control group should be performed and should be treated with the MPCA inactivated in such a way as to retain the structural integrity of the cell.
(iii) A control group in which the birds are dosed with sterile filtrate from production cultures should be performed concurrently with the test groups.

Test group

Number of birds per dosage level

Each treatment and control group shall contain at least 10 birds. When only one treatment group is tested, at least 30 birds shall be tested at that level.

Maximum hazard dosage level

(i)The highest oral dosage level tested is defined by the following formula:

maximum daily dose (units) = [MPCA] in TGAI × 5 mL/kg BW × weight of test bird (kg)

where

[MPCA] = concentration of MPCA

TGAI = technical grade of active ingredient

BW = body weight

(ii) When using injection routes, use 0.5 mL/kg BW for intravenous, and 2 mL/kg BW for intraperitoneal.

(iii) For MPCAs that produce a toxin, fractions of this dose should be calculated for lower doses. This dose is administered daily for 5 days. A justification shall be provided to support any reduction in the highest dosage level.

Treatment concentrations

A single group of birds may be tested at the maximum hazard dose. If deleterious effects, either due to toxicity or pathogenicity are observed, sequentially lower doses should be tested.

Method of Dose administration

Birds should be fasted for 12-15 hours overnight immediately prior to dosing. Fasting overnight prior to dosing for larger birds such as northern bobwhite and Japanese quail is commended. Shorter fasting periods of 2 hours are suitable for birds weighting around 50 gm or less. The test substance of volume not more than 10 ml/kg body weight is administered orally in a single dose by gavage or capsules. If Regurgitation happens, it should be properly recorded and possibly be reduced as it compromises the evaluation of toxicity.

Dosing regimen

Birds will receive oral doses daily for 5 days.

Determination of an LD50 or ID50

(i) The study endpoint must be chosen to reflect the activity of the specific microorganism under test, i.e. if an MPCA is expected to produce a toxin and has little or no infectivity, the appropriate endpoint would be death of the test organism. If, however, the major mechanism is pathogenicity, a more appropriate endpoint would be overt symptomatology.

(ii) The test data should establish that the avian oral LD50, defined as the dose required to kill 50 percent of the test organisms, or ID50, defined as the dose necessary to cause overt symptomatology in 50 percent of the test organisms, are greater than the hazard dosage level. If the LD50 or ID50 is lower than the maximum hazard dose, a definitive LD50 or ID50 with confidence limits should be established.

Observation period

Control and treated groups should be observed for at least 30 days after dosing initiation. If symptomatology or toxic signs are manifest at the 30th day, observation should continue until recovery, mortality, or unequivocal moribundity is established.

Experimental observations

1.Mean body weights for each test and control group at test initiation and weekly thereafter.
2.Total feed consumption for each test and control group at weekly intervals.
3.Method of test material preparation, concentration of the MPCA and total dose.
4.Amount of vehicle dosed per bird, if a vehicle other than water is used.
5.LD50 or ID50 in appropriate units with 95 percent confidence limits, if obtained.
6.Any signs of intoxication, abnormal behavior, and regurgitation, if any, occurs.
7. Reports of any pathogenic symptomatology or pathological changes.
8. Results of gross necropsies and histopathological findings conducted on enough birds to characterize any gross lesions including attempts, using appropriate techniques, to reisolate the MPCA from examined tissues.

Infectivity evaluation

Infectivity of the Microbial Pesticides is evaluated periodically during the test, and at the end of the test. For infectivity and persistence determinations, the microbial pesticide should be enumerated using selective culture medium technique for its presence in tissues, organs, body fluids, lesions and the injection site. Homogenized samples of liver, lungs, kidneys, brain, heart, spleen, blood, urine and faeces need to be cultured on selective medium to determine the infectivity/pathogenicity for Antagonistic bacteria/ Antagonistic fungi/Entomopathogenic fungi & Entomotoxic bacteria based microbial pesticide, whereas, in case of Baculoviruses based microbial pesticides, the presence of viable infective POB's should be detected using most sensitive respective larvae bioassay.

Result assessment

The safety of the test substance (microbial pesticide) should be based on the observations for its infectivity, pathogenicity, toxicity and mortality as the study end points.

Cautionary Statement

If any microbial pesticide shows infectivity or unusual persistence or significant signs of acute oral toxicity/pathogenicity etc. in any of the bird species, the applicant should –contact/consult toxicology expert of CIB&RC for further testing requirements.

Reference: OPPTS 885.4050; Avian Oral Tier I

Toxicity to earthworm

Objective: To assess and evaluate the toxic or pathogenic characteristics of a Microbial Pesticide in earthworm.

Purpose: The purpose of the earthworm toxicity study is to provide initial information on the toxicity of microbial pesticides. It provides information on health hazards likely to arise from exposure in earthworm.

Principle/Rationale: An evaluation of acute toxicity data should include the relationship, if any, between the earthworm's exposure to the test substance and the incidence and severity of all abnormalities. Mortalities and visible abnormalities related to appearance and behaviour are recorded. The concentrations to kill 50% of the earthworm (LC50) are determined. The screening test (filter paper contact test) involves exposing earthworms to test substances on moist filter paper in order to identify potentially toxic chemicals to earthworms in soil. The artificial soil test involves keeping earthworms in samples of a precisely defined artificial soil to which a range of concentrations of the test substance has been applied. Mortality is assessed 7 and 14 days after application. One concentration resulting in no mortality and one resulting in total mortality should be used.

Species: The recommended test species is *Eisenia foetida*.

Age: Worms should be adult (at least two months old with clitellum).

Weight: 300 to 600 mg.

Equipment and materials

Normal laboratory equipment and especially the following equipment and materials are necessary: – Earthworm cultures, Filter paper: 80 to 85 g/m2 , approximately 0.2 mm thick, medium grade – Artificial soil test substrate, for example, as follows: 10 per cent sphagnum peat (as close to pH 5.5 to 6.0 as possible, no visible plant remains, finely ground, dried to measured moisture content) 20 per cent kaolin clay (kaolinite content preferably above 30 per cent) 70 per cent industrial sand (fine sand should be dominant with more than 50 per cent of the particles between 50 and 200 microns) pH is adjusted to 6.0 ± 0.5 by addition of calcium carbonate.

The dry constituents are blended in the correct proportions and mixed thoroughly, either in a large-scale laboratory mixer or small electric cement mixer. Moisture content is then determined by drying a small sample at 105°C and re-weighing. Deionised water is added to give an overall moisture content of about 35 per cent of the dry weight, and the medium is thoroughly mixed. The complete mixture should be moist but not so wet that water appears when the artificial soil is compressed. With some peats a moisture content of over 35 per cent may be suitable.

All glass test containers, i.e. crystallising dishes or spoutless beakers, of approximately one litre covered with glass lids or perforated plastic film – An illuminated cabinet or chamber controllable to ± 2°C with a light intensity of 400 to 800 lux.

Filter paper test

Flat-bottomed glass vials approximately 8 cm in length and 3 cm in diameter are recommended. Their sides are lined with filter paper cut to a suitable size so it does not overlap appreciably.

The test substance is dissolved in water (if soluble up to a concentration of 1000 mg/l) or in a suitable organic solvent (e.g. acetone, hexane or chloroform), as appropriate, to give a range of known concentrations. One ml of solution is pipetted into each vial and evaporated to dryness under a slow stream of filtered compressed air, the vial being rotated horizontally as it dries (for substances that are relatively insoluble in either water or organic solvents this may have to be repeated several times to obtain the larger deposits required). The control vial should be treated with 1 ml of deionised water or appropriate organic solvent. After drying, 1 ml of deionised water is added to each vial to moisten the filter paper. Each vial is sealed with a cap or plastic film with a small ventilation hole.

A preliminary range-finding test may be done optionally prior to a more precise screening test. This could be done as follows:

Amount applied to filter paper	Concentration of solution applied
1.0 mg/cm^2	7×10^{-2} g/ml
0.1 mg/cm^2	7×10^{-3} g/ml
0.01 mg/cm^2	7×10^{-4} g/ml
0.001 mg/cm^2	7×10^{-5} g/ml
0.0001 mg/cm^2	7×10^{-6} g/ml

For the main screening test five or more treatment levels in a geometric series should be used. For each treatment, ten replicates, each consisting of one worm per vial, are the minimum requirement. More than one worm in a vial should not be used because the death of one worm may have adverse effects on others in the same vial. The precision of the test can be increased by using 20 replicates. In each test a range of treatment levels and ten control vials are used.

Worms should be kept on moist filter paper for three hours before being placed in test vials so they can void their gut contents. They are then washed and dried before use. During the test, vials are laid on their sides on trays. The test temperature is 20° ± 2°C. Tests are done in the dark and for a period of 48 hours with a further optional mortality assessment after 72 hours.

Worms are classified as dead when they do not respond to a gentle mechanical stimulus to the front end. Any behavioural or pathological symptoms should be reported.

Artificial soil test

A preliminary range-finding test before a more precise main test is optional here as well. It could be based on treatments in the range 0.01, 0.1, 1.0, 10, 100, 1000 mg/kg (dry weight of artificial soil). For the test proper, five concentrations in a geometric series are used.

The artificial soil plus test substance should, whenever possible, be made up as follows: immediately before the start of the test, an emulsion or dispersion of the test substance in deionised water is mixed with the artificial soil or sprayed evenly over it with a fine chromatographic or similar spray. If insoluble in water, the test substance can be dissolved in as small a volume as possible of a suitable organic solvent. The solvent should be allowed to evaporate. If the test substance is not soluble, dispersible or emulsifiable, 10 g of a mixture of fine ground quartz sand and quantity of test substance corresponding to 750 g wet weight of artificial soil are mixed with 740 g wet artificial soil for each test container. Only agents which volatilise readily may be used to solubilise, disperse or emulsify the test substance. The test medium must be ventilated before use. The amount of water evaporated should be replaced.The control should receive the same quantity of any additive agent.

For each test, 750 g weight of the test medium is placed into each glass container and ten earthworms, which have been conditioned for 24 hours in an artificial soil and then washed quickly before use, are placed on the test medium surface. The containers are covered with perforated plastic film to prevent the test medium from drying and kept under the test conditions for 14 days. Four replicates for each treatment are recommended. For each test, four control dishes, treated with the same solvent as that used in the test and containing ten worms, are used.

The test duration is 14 days (assessment of mortality at 7 and 14 days), and the test temperature is 20° ± 2°C. Testing is done in continuous light (to ensure that worms remain in the test medium throughout duration of test). The mortality is assessed by emptying test medium onto a glass tray or plate, sorting worms from the medium and testing their reaction to a mechanical stimulus at the front end. After the 7-day assessment worms and medium are replaced in the test container. Any behavioural or pathological symptoms noted should be reported.

At the end of the test the moisture content of the test medium should be assessed and reported.

Experimental Observation

i)Average live weight and number of live worms per treatment at start and end of test.
ii)Description of obvious physical or pathological symptoms or distinct changes in behaviour observed in the test organisms.

iii)Mortality in control animals and mortality with reference and test substance.

iv)Moisture content of artificial soil at start and at end of test, pH value at start of test.

v)The highest concentration causing no mortality and the lowest concentration causing 100 per cent mortality.

Result assessment

The safety of the test substance (microbial pesticide) should be based on the observations for its mortality and any behavioral abnormality as the study end points. Lethal Concentration (LC 50) should be calculated.

Reference: OECD 207, Earthworm, Acute Toxicity Tests

Toxicity to fresh water fish

Objective: To assess and evaluate the toxic or pathogenic characteristics of a Microbial Pesticide in fresh water fish species.

Purpose: The purpose of the fresh water fish toxicity is to provide initial information on the toxicity of microbial pesticides. It provides information on health hazards likely to arise from exposure by the oral route in fish.

Principle/Rationale: An evaluation of acute toxicity data should include the relationship, if any, between the fish's exposure to the test substance and the incidence and severity of all abnormalities. Mortalities and visible abnormalities related to appearance and behaviour are recorded. The concentrations to kill 50% of the fish (LC50) are determined.

Species: Testing shall be performed on one fish species, preferable the rainbow trout if the microbial pest control agent (MPCA) has only a terrestrial use and direct aquatic exposure is not expected, or two fish species, preferably the bluegill sunfish and rainbow trout, when direct aquatic exposure is anticipated. Other species of fish may be used, but a justification must be supplied based on an increased susceptibility to the MPCA or ecological considerations that preclude the use of recommended species.

Age: Testing of young fish is preferred. The fish should be of the same age and have normal appearance. Fish should be juveniles and originate from the same source and population to ensure uniformity.

Weight: Fish should weigh between 0.5 and 5.0 g and be from the same year class. The length of the longest fish should be no more than twice that of the shortest fish.

Holding of Fish:
All fish should be held in the laboratory for at least 9 days before they are used for testing. The first 48 hours constitute a settling-in period. Then, fish should be acclimatised for at least 7 days (48 hours settling-in + 7 days acclimatisation = 9 days) in water similar to test immediately before the start of the test. Holding of fish should be under the following conditions:

- Photoperiod: appropriate to the species
- Temperature: appropriate to the species
- Oxygen concentration: at least 80% of air saturation value

- Feeding: three times per week or daily until 24 - 48 hours before the exposure is started. Feed may be given to satiation. Surplus food and faeces should be removed as necessary to avoid accumulation of waste.

Conditions of Exposure

Loading: for freshwater fish, maximum loading of 0.8 g wet weight fish/L for static and semi-static renewal testing is recommended. For flow-through systems, the recommended maximum loading is 0.5 g wet weight fish/L per 24 hours

Light: should be within the photoperiod ranges specified for the test species and with an intensity of 10-20 μE/m2 /s, 540-1000 lux, or 50-100 ft-c.

Temperature: the water temperature should not differ by more than 2°C between test vessels or between successive days at any time during the exposure.

Oxygen concentration: not less than 60% of the air saturation value.

Groups and numbers of fish required:

Control group

i)A negative, non dosed control group should be run concurrently the test groups.
ii) A control group in which the fish are exposed to sterile filtrate from production cultures should be performed concurrently with the test groups.

Treated group

Ten fish per group should be used in multiple group testing, 30 fish in single group testing.

Route of exposure

i) The test substance shall be administered as a suspension directly into the water (i.e. aqueous exposure).
ii) Additionally, the MPCA should be administered through the oral route of exposure, preferably through incorporation in standard fish food or through the use of infected insects.

Maximum hazard dose

i)At a minimum, the concentration in the test water (for aqueous exposure) should, whenever possible, be at least 106 units/mL or at least 1,000× the maximum calculated pesticide concentration in a 6–in layer of water immediately following a direct application to a 6–in layer of water, whichever is greater and attainable. Measures should be taken to ensure that the initial concentration of the MPCA is maintained throughout the test should be described.

ii)Feed used in the dietary exposure should be supplemented with the test substance to achieve a microbial concentration per gram of food of at least 100× the calculated cell density per milliliter in a 6–in layer of water immediately following a direct application to a 6–in layer of water.

Treatment concentrations

A single, group of fish may be tested at the maximum hazard dose. If deleterious effects, due either to toxicity or pathogenicity are observed, sequentially lower doses should be tested.

Determination of LC50 or ID50

i) The study endpoint must be chosen to reflect the activity of the specific microorganism under test, i.e. if an MPCA is expected to produce a toxin and has little or no infectivity, the appropriate endpoint would be mortality. If, however, the major mechanism is pathogenicity, a more appropriate endpoint would be overt symptomatology.

ii)The data should establish that the freshwater fish LC50, defined as the dose required to kill 50 percent of the test organisms, or IC50, defined as the dose necessary to produce overt symptomatology in 50 percent of the test organisms, is greater than the maximum hazard dosage level. If the LC50 or IC50 is lower than the hazard dose, an LC50 or IC50 with confidence intervals should be established.

Duration of test

Control and treated groups should be observed for 96 hours after dosing initiation.

Experimental observations

A minimum of 2 observations should be conducted within the first 24 hours of the study with preferably at least 3 hours between observations. Fish could be inspected at 2 ± 0.5 h, 5 ± 1 h and 24 ± 2 h after the start of the exposure (day 0-1). On days 2-4 of the test, all vessels with living fish should be inspected twice per day (preferably early morning and late afternoon to best cover the 24-hour periods). Mortalities and visible abnormalities in regard to equilibrium (e.g. loss of balance, head up or down, floating at surface or sinking), appearance (weak or dark pigmentation, exophthalmia), ventilatory behaviour (e.g. hyper, hypo or irregular ventilation, coughing) and swimming behaviour (hyper or hypo activity, immobility, convulsions, near surface or bottom, dense or loss of schooling) are recorded.

Humane killing of fish: Surviving fish of the treatment groups are euthanised at the end of the exposure, whereas euthanasia of surviving control fish is not required, but they should not be used in another test.

Result assessment

i)Loading (weight of organisms per unit volume of medium).
ii)Cumulative mortality at each concentration at the recommended observation times;
mortality in the control(s).
iii)Percentages of test animals that died or showed symptomology
iv)The LC50 values at 24, 48, 72 and 96 hours with 95% confidence limits,
v)The slope of the concentration-response curve after 96 hours exposure,
vi)Graph of the concentration-mortality curve at the end of the exposure,
vii)Incidence and description of visible abnormalities observed during exposure, additional clinical signs can be recorded.

viii) Incidents in the course of the test which might have influenced the results.

ix) Description of the statistical methods used and treatment of data (e.g. probit analysis, logistic regression model, arithmetic or geometric mean for LC50 values, time weighted average.

References: OPPTS 885.4200 Freshwater Fish Testing, Tier I

OECD Test Guideline No. 203 Fish, Acute Toxicity Testing

Honey bee toxicity study

Objective: To assess and evaluate the toxic or pathogenic characteristics of a Microbial Pesticide in honey bees.

Purpose: The purpose of the honey bee toxicity is to provide initial information on the toxicity of microbial pesticides. It provides information on health hazards likely to arise from exposure by oral as well as contact in honey bees.

Principle/Rationale: Adult worker honeybees are exposed to a range of doses of the test substance dissolved in appropriate carrier, by direct application to the thorax in form of droplets. The test duration is 48h. If the mortality rate is increasing between 24 and 48h whilst control mortality remains at an accepted level, i.e. ≤10%, it is appropriate to extend the duration of the test to a maximum of 96h. Mortality is recorded daily and compared with control values. The results are analysed in order to calculate the LD50 at 24 and 48h, and in case the study is prolonged at 72h and 96h.

Test species:
Testing shall be performed on the honey bee, *Apis mellifera.*

Age: Young, adult worker bees of the same race should be used, i.e. bees of similar age, feeding status. When the MPCA may be expected to affect insect larvae, test insects should include honey bee larvae.

Housing and Feeding Conditions:
Temperature: 25 ± 2 °C
Relative Humidity: 50-70 %, (However, humidity should not be below 30% or exceed 70% at any given point of time).
Lighting: 12 hours light and dark cycle
Diet and water: *Ad libitum* standard diet, (specific to the species) and filtered water (free from contamination).
Acclimatization: The bees should be acclimatized to the laboratory conditions for at least 1 day prior to dosing by keeping them in individual cages.

Description of method

Collection of bees
Bees should be obtained from adequately fed, healthy, as far as possible disease-free and queen-right colonies with known history and physiological status. They could be collected in the morning of use or in the evening before test and kept under test conditions to the next day. Bees collected from frames without brood are suitable. Collection in early spring or late autumn should be avoided, as the bees have a changed physiology during this time. If tests have to be conducted in early spring or late autumn, bees can be emerged in an incubator and reared for one week with “bee bread” (pollen collected from the comb) and sucrose solution. Bees treated with chemical substances, such as antibiotics, anti-varroa, etc., should not be used for toxicity test for four weeks from the time of the end of the last treatment.

Test cages
Easy to clean and well-ventilated cages are used. Any appropriate material can be used, e.g. stainless steel, wire mesh, plastic, disposable wooden cages, etc. Groups of ten bees per cage are preferred. The size of test cages should be appropriate to the number of bees, i.e. providing adequate space.

Handling and feeding conditions
Handling procedures, including treatment and observations may be conducted under (day) light. Sucrose solution in water with a final concentration of 500 g/l (50% w/v) should be used as food and provided *ad libitum* during the test time, using a bee feeder. This can be a glass tube (ca 50 mm long and 10 mm wide with the open end narrowed to about 2 mm diameter).

Preparations of bees
The collected bees may be anaesthetized with carbon dioxide or nitrogen for application of the test substance. The amount of anaesthetic used and times of exposure should be minimised. Moribund bees should be rejected and replaced by healthy bees before starting the test.

Preparation of doses

If the test substance is a water miscible compound this may be dispersed directly in 50 per cent sucrose solution. For technical products and substances of low water solubility, vehicles such as organic solvent, emulsifiers or dispersants of low toxicity to bees may be used (e.g. acetone, dimethylformamide, dimethylsulfoxide). Two separate control groups should be used: a solution in water, and a sucrose solution with the solvent/carrier at the concentration used in dosing solutions.

Note: If an organic solvent or a wetting agent is used three additional control batches of each ten bees for the solvent or the wetting agent have to be included.

Test and control groups

Control group: A minimum of three control batches, each of ten bees. If an organic solvent or a wetting agent is used three additional control batches of each ten bees for the solvent or the wetting agent have to be included.

Test group: The number of doses and replicates tested should meet the statistical requirements for determination of LD50 with 95% confidence limits. Normally, five doses in a geometric series, with a factor not exceeding 2.2, and covering the range for LD50, are required for the test. However, the number of doses have to be determined in relation to the slope of the toxicity curve (dose versus mortality) and with consideration taken to the statistical method which is chosen for analysis of the results. A range-finding test enables the choice of the appropriate doses.
A minimum of three replicate test groups, each of ten bees, should be dosed with each test concentration.
Control and treated bees should be observed for at least 30 days after dosing.

Toxic standard
A toxic standard should be included in the test series. At least three doses should be selected to cover the expected LD50 value. A minimum of three replicate cages, each containing ten bees, should be used with each test dose. The preferred toxic standard is dimethoate for which the reported contact LD50-24h is in the range 0.10-0.30 µg a.i./bee.
However, other toxic standards would be acceptable where sufficient data can be provided to verify the expected dose response (e.g. parathion).

Administration of doses
For oral test: Each test group of bees should be provided with 100-200 µl of 50% sucrose solution in water, containing the test substance at the appropriate concentration. Once consumed (usually within 3-4 hours or 6 hours for some test substance or at higher concentration), the feeder should be removed from the cage and replaced with one containing sucrose solution alone.

For contact test: Anaesthetized bees are individually treated by topical application. The bees are randomly assigned to the different test doses and controls. A volume of 1 µl of solution containing the test substance at the suitable concentration should be applied with a microapplicator to the dorsal side of the thorax of each bee. Other volumes may be used, if justified. After application, the bees are allocated to test cages and supplied with sucrose solutions.

Limit Test
In case it is expected that pesticide has low bee toxicity, limit test can be preferred using the same procedure with dose level of 100µg a.i /bee. If no mortality are encountered at a dose of 100 µg (active ingredient)/bee, then the LD50 is greater than the limit test dose & the higher dose need not to be tested except when human exposure indicates the need for a higher dose level to be used. Full study should be conducted if any mortality occur.

Duration of test

For oral test: The duration of the test is 48 hr after the test solution has been replaced with sucrose solution alone. If mortality continues to rise by more than 10% after the first 24 hr, the test duration should be extended to a maximum of 96 hr provided that control mortality does not exceed 10%.

For contact test: The duration of the test is 48h. If mortality increases by more than 10 per cent between 24h and 48h, the test duration should be extended up to a maximum of 96h provided that control mortality does not exceed 10 per cent.

Experimental observations
For oral test:
i)Mortality is recorded at 4 hr after start of the test and thereafter at 24 hr and 48 hr (i.e. after given dose). If a prolonged observation period is required, further assessments should be made at 24 hours intervals, upto a maximum of 96 hour, provided that the control mortality does not exceed 10%.

ii)The amount of diet consumed per group should be estimated. Comparison of the rates of consumption f treated and untreated diet within the given six hours can provide information about palatability of the treated diet.
iii)All abnormal behavioral effects observed during the testing period should be recorded.

For contact test:
i)Mortality is recorded at 4 h after dosing and thereafter at 24h and 48 h. If a prolonged observation period is required, further assessments should be made at 24 hours intervals, to a maximum of 96 hours, provided that the control mortality does not exceeding 10 per cent.
ii)All abnormal behavioural effects observed during the testing period should be recorded.

Result assessment
The safety of the microbial pesticide should be based on the observations for its mortality and abnormal behavioural effects observed as the study end points.

References: OECD 214: Honeybees, Acute Contact Toxicity Test

Toxicity to silkworm

Objective: To assess and evaluate the toxic or pathogenic characteristics of a Microbial Pesticide in silkworm.

Purpose: The purpose of toxicity study to silkworm is to provide initial information on the toxicity of microbial pesticides. It provides information on health hazards likely to arise in silkworm.

Principle/Rationale: To evaluate the effects of a microbial pesticide on silkworms. Silkworms should be exposed to the pesticidal microbe in a high concentration, and thereby the effects on test silkworms should be examined.

Test animals:

Species: Testing shall be performed on silkworm, *Bombyx mori*.

Age: Fourth instar larvae of silkworm just after moulting.

Housing and Feeding Conditions:
Temperature: 25 ± 2 °C
Relative Humidity: 50-70 %, (However, humidity should not be below 30% or exceed 70% at any given point of time).
Lighting: 12 hours light and dark cycle
Diet and water: Ad libitum standard diet, (specific to the species) and filtered water (free from contamination).
Acclimatization: The silkworms should be acclimatized to the laboratory conditions for at least 1 day prior to dosing by keeping them in individual cages.

Test and control groups:

Control group:

Untreated group (When a wetting agent is used in the treated group, it should also be used in the untreated group.)

Treated group:

The concentration used should be 10 times the maximum concentration to be shown in the application for registration of the microbial pesticide concerned. When any effect has been observed at the above concentration, a dose-response test should be performed to clarify the concentration at which an effect is seen. The number of test silkworms used should be 50 per test group, and the test should be performed twice per test group.

Exposing method and raising conditions

Control group:

Mulberry leaves not contaminated with the pesticidal microbe, or artificial diet (not containing antibiotic) should be given to the silkworms every day.

Treated group:

Where the pesticidal microbe involved is a filamentous fungus, the silkworms should be immersed in its suspension. After that, the silkworms should be given mulberry leaves every day that are not contaminated with the pesticidal microbe. To make the microbe easily stickable to the silkworms, a wetting agent may be added to the suspension.

Where the microbe involved is one other than filamentous fungus, mulberry leaves immersed in a suspension of the pesticidal microbe and then dried, or artificial diet mixed with the said suspension (0.05 - 0.1 ml/g) should be given to the silkworms for 24 hours. After that, the silkworms should be given untreated mulberry leaves or artificial diet every day. The artificial diet to be selected should be one that the silkworms like to eat and is good for their growth.

Test period: Test period should be for 20 days after administration.

Experimental observations

i) Observation of signs and symptoms

The number of dead silkworms should be checked every day, and when necessary, such items as days of 4th and 5th instar, number of cocoon-spinning silkworms, pupation rate, weight of cocoon, weight of cocoon layers and symptom of poisoning should also be observed. All abnormal behavioural effects observed during the testing period should be recorded.

ii) Pathological examination

When a silkworm has died in the course of the test, it should be examined as to the presence of any infection etc. caused by the pesticidal microbe.

Result assessment

The concentrations to kill 50% of the silkworm (LC50) are determined. The safety of the microbial pesticide should be based on the observations for its toxicity and mortality as the study end points.

References: Guidelines for preparation of data necessary for safety evaluation of microbial pesticides, The notification No. 9-Seisan-5090, issued on August 29, 1997 by the Director, Plant Protection Division, Agricultural Production Bureau, Ministry of Agriculture, Forestry and Fisheries, Japan.