Effective Inoculation Method and Optimum Concentration of *Oryctes* Virus to Infect *Oryctes rhinoceros* Adults

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Abstract
This research was carried out to determine the effective inoculation method and optimum concentration of local Oryctes virus (OrV) for successful infection of Oryctes rhinoceros (L.) adults in laboratory which released to the field to spread the viral disease among healthy larvae and beetles. Percentage of infected beetles were significantly different (P<0.001) among the two methods of inoculation and untreated control. After 21 days of inoculation, oral introduction method, swim method and control recorded 88.8%, 44.4% and 11.1% of OrV infection respectively indicating the most effective inoculation method as oral introduction. Cumulative percentage mortality of O. rhinoceros adults with 10^1ppm, 10^2ppm, 10^3ppm, 10^4ppm and 10^5ppm concentrations were recorded as 7.3%, 25.1%, 33.3%, 81.4% and 100% respectively. LC_{50} was $10^{2.7}$ppm and LT_{50} for 10^4ppm and 10^5ppm concentrations were 23 days and 12 days respectively. Best concentration to oral inoculation of O. rhinoceros with OrV for field release was selected as 10^4ppm

Key words: *Oryctes rhinoceros*, *Oryctes* virus, Lethal concentration, Lethal time

1. Introduction

The black beetle *Oryctes rhinoceros* (Linnaeus) is a major pest occurring in all coconut growing areas of Sri Lanka. Palms with 50% of frond damage corresponded to leaf area reduction of 13% and decrease in nut yields by 23% as compared to normal palms. Attack in young seedlings may out rightly kill them. It may also provide entry points for lethal secondary attacks by the red weevils or by pathogens.

In 1966 a virus disease of black beetle was discovered by Huger in Malaysia and this pathogen has recently been placed under its own virus category *Oryctes.* Adult stage and larval stage are susceptible to diseases. Midgut epithelial cells of virus infected beetles are the main sites of virus replication. Many gut cells die from the infection and gut swells with milky content. The adults after taking up infection become lethargic, short lived (25 days of 70) and reduced the egg laying capacity in 90% (Zelazny, 1978). Infected beetles act as a flying virus vectors which ingested the virus to healthy larvae and beetles by feeding, excreta and at breeding sites (Zelazny, 1976). Five percent of the grubs and 12% of the adults had natural

virus infection. But that natural transmission enables it to persist in nature and reduce the beetle population. Therefore beetles which were inoculated with the virus under laboratory condition released to field as biological control method.

Different methods have been used to inoculate the virus for infect beetles. Most frequently used method is letting beetles to swim in a suspension of virus. Feeding beetles with a virus suspension prepared from beetle gut is another method. At the end of this experiment expected to find the most suitable inoculation method, lethal dosage and lethal time of virus inoculum and optimum concentration.

2. Materials and method

The research study was conducted at the laboratory of Crop Protection Division, Coconut Research Institute (CRI), Lunuwila and the experimental period was six months.

2.1. Collection of beetles

Beetles were collected from pheromone traps installed in plantations which haven't been released with *Oryctes* virus infected beetles. They were kept separately in plastic bottles filled with sterilized coir dust and ripen banana pieces were given as feeding materials.

2.2. Identification of virus infection

Beetles were dissected to determine the virus infection by observing the midgut symptoms. Virus infected beetles has swollen, whitish mid gut with white mucous fluid (Zelazny, 1978). Further confirmations of virus infection were done by staining the midgut contents using Giemsa staining method. The gut contents of healthy beetles usually contain no cells, except for few small pieces of tissues while infected guts contain many mid gut epithelial cells which were stain in pink colour (Goric, 1980).

2.3. Preparation of inoculum

Mid guts of virus infected beetles stored in the refrigerator were used for the preparation of virus inoculum. One gram of infected guts was ground and add 100ml of 5% sucrose solution to prepare a 10^4ppm virus inoculum.

2.4. Identification of effective inoculation method

Experiment consists of two treatments, one control trial with three replicates. A group of 432 beetles of approximately same age was separated by observing the amount of hairs remaining on the two posterior ventral segments of the abdomen that worn out with age (Cumber, 1957). One group of 108 beetles were infected with *Oryctes* virus by placing 0.1ml of 10^4ppm virus inoculum on beetles' mouth using a pasture pipette and allow to suck up. Another group of 108 beetles were allowed to swim in 100ml of 10^4ppm virus suspension for 10 minutes. The third group of 108 beetles was kept uninfected as control

2.4.1. Data collection and analysis

Six beetles were randomly selected from each replicate of the treatments and control at 2,5,8,11,14 and 21 day intervals for dissection. The number of beetles infected with virus was determined. The invariate analysis of variance for mean percentage infection was performed at 5% probability level by using.

2.5. Identification of optimum concentration

A group of 180 beetles of approximately the same age was collected by observing the amount of hair remaining on the two posterior ventral segments of the abdomen (Cumber, 1957) and they were randomly assigned in to 6 groups of 30 beetles. Five concentrations of 10^1ppm, 10^2ppm, 10^3ppm, 10^4ppm and 10^5ppm of virus inoculum were prepared by mixing 5% sucrose solution and infected guts in following amounts (Table 1) Experiment had 5 treatments which were represent each concentration and uninfected control. Thirty beetles per concentration were inoculated by placing 0.1ml of virus suspension on beetles' mouth and allowed to suck up. Thirty beetles were kept uninfected as control and they were fed with 5% sucrose solution only.

2.5.1. Data collection and analysis

Number of dead beetles due to virus infection in each concentration was counted at 24 hour intervals during the experimental period of 38 days. Cumulative percentage mortality of treatments was corrected by using Abbott's formula. Transform the concentration to log concentration and corrected percentage mortality to probit mortality and graphs were plotted to determine the LC_{50} value and LT_{50} value. Data were analysis by using the CATMOD procedure.

3. Results and Discussion

Oryctes rhinoceros adults were infected by both oral and swim method of inoculation. The percentage of infected beetles increased up to 11^{th} day of inoculation at a higher rate and thereafter at a low rate (Table 2) in both oral inoculation and swim method of inoculation. After getting entry, OrV reaches the nuclei of midgut epithelial cells of adults where it replicates. In the four hour post infection period virus absorption in to the plasma membrane and uptake in cytoplasmic vesicles occurs. Seven to 12 hours post infection, viral replication in the clear area of the hyper trophied nucleus occurs and finally, at 16 hours or more virus release from plasma membrane take place and it takes at least more than 2 days to show infection symptoms (Crawford and Sheehan, 1984). Results of this story also agree with above observations and it took 5 to 11 days for *Oryctes rhinoceros* adults to show the viral symptoms.

A significantly higher ($P < 0.001$) percentage of infected beetles were recorded in the oral inoculation method than the swim method at all the time periods (Table 2). In the oral inoculation method, the suspension dropped in to the mouth of the beetle is forced to imbibe by them and also the amount of virus injected by each is known. In the swim method, the amount injected by each beetle may vary and they may not ingest sufficient amount of OrV to prone an infection. The only disadvantage of the oral method of inoculation that it is time consuming because each beetles should be inoculate individually. Zelazny *et al* (1987) in Davao Research Centre Indonesia revealed, submerging the beetles in a virus suspension resulted in a very low rate of infection (28%) while inoculating beetles by placing a small drop of virus inoculum on there mouth produce infection (69%) more consistently. Another study of (Zelazny and Alfiler, 1986) at Albay Research Institute Indonesia recorded that submerging beetles in suspension of virus killed larvae cause low rate of OrV infection (24%) while inoculating beetles by placing a small drop of virus containing cell culture fluid on there mouth recorded 76% of infection.

All concentrations of virus inoculum tested caused mortality in black beetle adults. The percentage mortality

increased with the increasing concentration of OrV inoculum. There was no significant difference in cumulative percentage mortality in concentrations of 10^1ppm to 10^3ppm. They were significantly lower ($P < 0.004$) than the mortality recorded in concentration of 10^4ppm and 10^5ppm. Also cumulative percentage mortality between 10^4ppm and 10^5ppm were significantly different ($P < 0.02$). At 10^5ppm concentration all beetles were dead (Table 3). Concentrated preparations of cell culture fluid were tested as 10^0ppm, 10^1ppm, 10^2ppm and 10^3ppm dilutions in 10% sucrose by Zelazny *et al*, (1987) in Philippines. Percentage infection by cell culture fluids of 10^0ppm, 10^1ppm, 10^2ppm and 10^3ppm were recorded as 10%, 35%, 47% and 63% respectively. Zelazny *et al*, (1987) used the virus inoculum derived from *Heteronychus arator* cell culture. In present study, virus inoculum was derived from guts of infected beetles. By comparing the results of both research can revealed that the cell culture fluid gave higher rate of infection than the virus inoculum prepared by guts of infected beetles in present study. LC_{50} is the concentration of virus inoculum required to kill 50% of the beetle population. In present study the LC_{50} of the local OrV inoculum was $10^{2.7}$ppm (Figure 4). Concentration that kills 90% of pest population known as LC_{90} and it is defined mainly for microbial insecticides. Concentration of OrV inoculum that kills 90% of *Oryctes rhinoceros* population was $10^{4.1}$ppm (Figure 4).

LT_{50} is the time required to kill 50% of a pest population. Fifty percent mortality was not achieved in concentration 10^1ppm, 10^2ppm, 10^3ppm during the 38 days of experimental period. The concentrations of 10^4ppm and 10^5ppm recorded LT_{50} as 23 days and 12 days respectively (Figure 5).

The objective of releasing laboratory infected beetles of OrV to coconut fields is spreading the disease among healthy beetles and larvae. Therefore, those beetles should act as successful flying virus vectors that effectively spread the disease. Activities of those beetles as effective virus vectors are mainly determined by the inoculation method and concentration of virus inoculum ingested by them in the laboratory. The infected beetles should be able to remain in the field for a reasonably long period to infect the field population. Although the concentration of 10^5ppm gave 100% mortality it kills 50% of the beetle population in 12 days. Virus inoculum of 10^4ppm kills less number of beetles (81.4%) but it takes 23 days to kill 50% of the beetle population. Therefore oral inoculation of *Oryctes rhinoceros* adults with 10^4ppm OrV inoculum could be taken as effective concentration to infect *Oryctes rhinoceros* in laboratory.

4. Conclusions

Oral feeding method is the best inoculation method of OrV suspension to infect *Oryctes rhinoceros* adults in laboratory conditions. After inoculation of OrV suspension to *Oryctes rhinoceros* adults it takes 5 to 11 days to show viral symptoms. Lethal concentration (LC_{50}) was $10^{2.7}$ppm. Lethal time (LT_{50}) for 10^4ppm and 10^5ppm virus inoculum was 23 days and 12 days respectively. Virus inoculum of 10^4ppm is the effective concentration for infect *Oryctes rhinoceros* adults in laboratory condition for field release.

Table 1: Weight of guts and amount of 5% sucrose solution used to prepare each concentration

Concentration	Weight of guts	Volume of 5% sucrose solution
10^1ppm	1mg	100ml
10^2ppm	10mg	100ml
10^3ppm	100mg	100ml
10^4ppm	1000mg	100ml
10^5ppm	10000mg	100ml

Table 2: Mean percentage OrV infection with respected to time in two inoculations methods and control

	Mean % infection ± SE		
	Oral	Swim	Control
5 days	27.7 ± 0.054^A	5.5 ± 0.054^B	5.5 ± 0.054^B
8 days	61.1 ± 0.054^A	16.6 ± 0.054^B	11.1 ± 0.054^B
11 days	83.3 ± 0.054^A	38.8 ± 0.054^B	16.6 ± 0.054^C
14 days	83.3 ± 0.054^A	44.4 ± 0.054^B	11.1 ± 0.054^C
21 days	88.8 ± 0.054^A	44.4 ± 0.054^B	11.1 ± 0.054^C

* SE – Standard Error. Means followed by same letters are not significant

Table 3: Cumulative percentage mortality of O. rhinoceros beetles at different concentrations of virus inoculum.

Concentration	Cumulative percentage mortality ± SE
10^1ppm	7.3 ± 0.27[a]
10^2ppm	25.1 ± 0.83[a]
10^3ppm	33.3 ± 0.46[a]
10^4ppm	81.4 ± 0.40[b]
10^5ppm	100.0 ± 0.39[c]

* SE – Standard Error. Means followed by same letters are not significant

Figure 4: Changing of probit mortality with log concentration

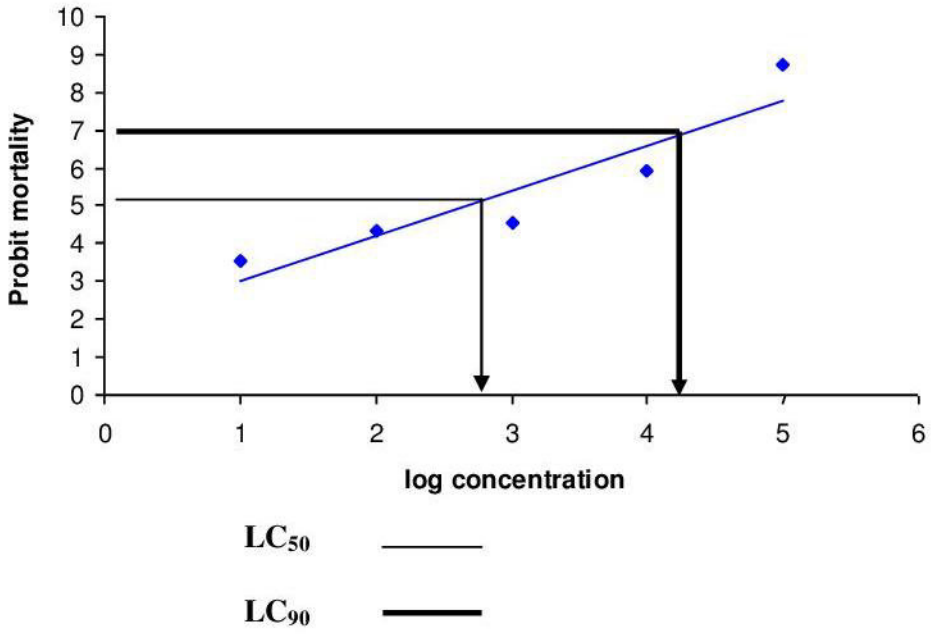
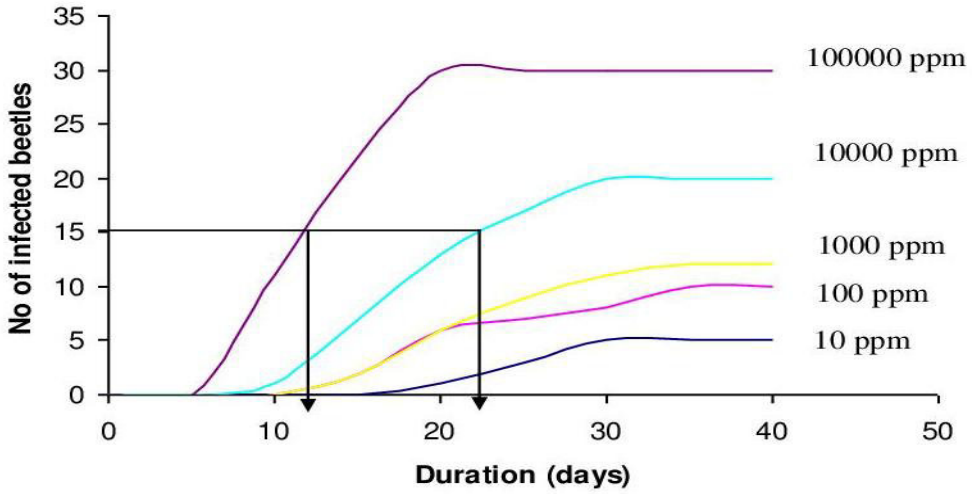


Figure 5: No of death beetles with respect to time in five virus inoculums



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