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RESPONSE OF *ORYCTES RHINOCEROS* LARVAE TO INFECTION BY *ORYCTES* BACULOVIRUS*

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ABSTRACT

Three larval instars of *Oryctes rhinoceros* (L.) were infected with *Oryctes* baculovirus through contaminated feed. The primary external pathological symptom was translucency of the thorax. Internally, the midgut contained turbid fluid with flakes of sloughed-off epithelial tissue. Baculovirus infection in larvae could be diagnosed in Giemsa stained scrappings of the midgut epithelium, two days after inoculation, whereas bioassay test could detect infection on the third day. Median lethal dose (LD_{50}) and weight of infected tissue containing one LD_{50} dose for all the three instars were computed. The second instar was the least susceptible to virus infection. The order of susceptibility among the instars was I>III>II. Translucency was found to appear 3-4 days prior to death. Lethal infection period ranged from eleven to sixteen days. The thermal inactivation point of the virus in tissue suspension was at 56°C.

INTRODUCTION

The biological suppression of the coconut rhinoceros beetle, *Oryctes rhinoceros* (L.), brought about by *Oryctes* baculovirus, is a major achievement in successful biological control (Caltagirone, 1981). The virus was first recorded causing a disease in *O. rhinoceros* larvae in Malaysia (Huger, 1966), and was subsequently introduced into many of the pest-ravaged South Pacific Islands (Marschall, 1970; Hammes, 1971; Zelazny, 1973, 1977a; Young, 1974; Bedford, 1976, 1977). The virus disease spread rapidly, established and reduced populations of the pest below the economic injury level and the control persisted for a number of years (Bedford, 1980). The natural occurrence of the virus in the pest population has also been reported from the Philippines, Indonesia and India (Zelazny, 1977b; Mohan, Jayapal and Pillai, 1983). The effect of virus infection (Malaysian isolate) on the larvae of *O. rhinoceros* has been described by Zelazny (1972). This publication describes the sequence of pathological changes occurring in the larval midgut of *O. rhinoceros* due to viral infection.

MATERIALS AND METHODS

Source of virus inoculum

The virus stock used in this experiment was obtained from virus infected midguts of field-collected *Oryctes* adults and passaged in healthy, third instar

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Oryctes grubs. Diseased larvae, just prior to death were stored at −20°C. Whenever virus inoculum was required, the frozen grubs were thawed quickly in warm water (40°C) and triturated in chilled phosphate buffer (0.05M, pH 8.0 with 0.5M, EDTA) in a blender.

Effect of infection on the midgut of grubs

To study the pathological changes in the midgut of virus-infected grubs, fifty healthy middle third instar grubs were infected by introducing them into a large plastic box containing autoclaved cattle dung as feed, mixed with 10% virus inoculum (grams, infected tissue in buffer). An equal number of grubs was maintained on virus-free feed as control. Five grubs each from the treated and control groups were picked up every day at random, washed in running tap water, anaesthetised with ether and dissected along the dorsal plane. Care was taken to avoid any mechanical damage to the swollen gut. The fore-and midgut were slit dorsally and exposed. Midgut fluid, if present, was aspirated. The midgut epithelium was gently scraped with the edge of a microscope slide and suspended in minimum volume of phosphate buffer. A smear of the tissue suspension was made, air dried, fixed in methanol for 3–4 min and stained in Giemsa for one hour (Lillie, 1965), washed in distilled water and examined. The following external and internal pathological symptoms of the infected grubs were looked for and recorded :

External : Presence / absence of: a) translucency or waxy appearance and b) extrovertion of rectum.

Internal : Presence / absence of: a) feed in the midgut and b) fluid with flakes of sloughed-off midgut epithelial tissue.

The midgut fluid was used for the detection of virus particles by electron microscopy. The aspirated fluid was made upto 10 ml in phosphate buffer and clarified by low speed centrifugation at 10, 000g for 20 min, followed by a high speed centrifugation at 65, 000 g for one hr in a Beckman ultracentrifuge, model L5 50B. The pellet was resuspended in 0.2 ml buffer and was applied on to carbon-coated copper grids and negatively stained with 2% phosphotungstic acid.

Bioassay of the sample grubs was done as described by Zelazny (1972).

Median lethal dose (LD_{50}) in grubs of different instars

Acutely diseased grubs were weighed and triturated in chilled phosphate buffer. Log dilutions of the triturate in buffer (10^{-2} to 10^{-5}) were made and one ml of each dilution was added to 100g of autoclaved cattle-dung (with 50% moisture) in individual plastic boxes and mixed. Grubs used in this experiment were laboratory reared and disease-free. A healthy grub of appropriate instar was introduced into each box. Ten grubs per log dilution were used. Each replication comprised either the first, second and middle third instar grubs. Each instar was exposed to log dilutions of 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} and an untreated control. Treatments were replicated three times. The grubs were examined twice a week for the appearance of

disease symptoms, especially translucency of the dorsal side. Death of grubs with typical symptoms was noted and median lethal dose and standard errors were calculated as described by Reed and Muench (1938).

Thermal inactivation point

Twenty ml aliquots of 10^{-1} log dilution of triturated diseased tissue in buffer, were held in thin walled glass tubes and subjected to different temperatures as 48, 50, 52, 54, 56, 58, 60 and 65°C for ten minutes in a constant temperature water bath with stirrer (Noordan, 1973). One ml of the heat treated suspension was added per box containing 100g of autoclaved cattle dung. Healthy, middle third instar grubs were used for bioassay. Twenty grubs were exposed to viral suspension held at one particular temperature and two replications were conducted. A negative control group comprised of twenty grubs was treated with buffer alone and a positive control group was treated with viral suspension (10^{-1} dilution) without heat treatment. Observations for symptoms of viral disease were recorded twice a week.

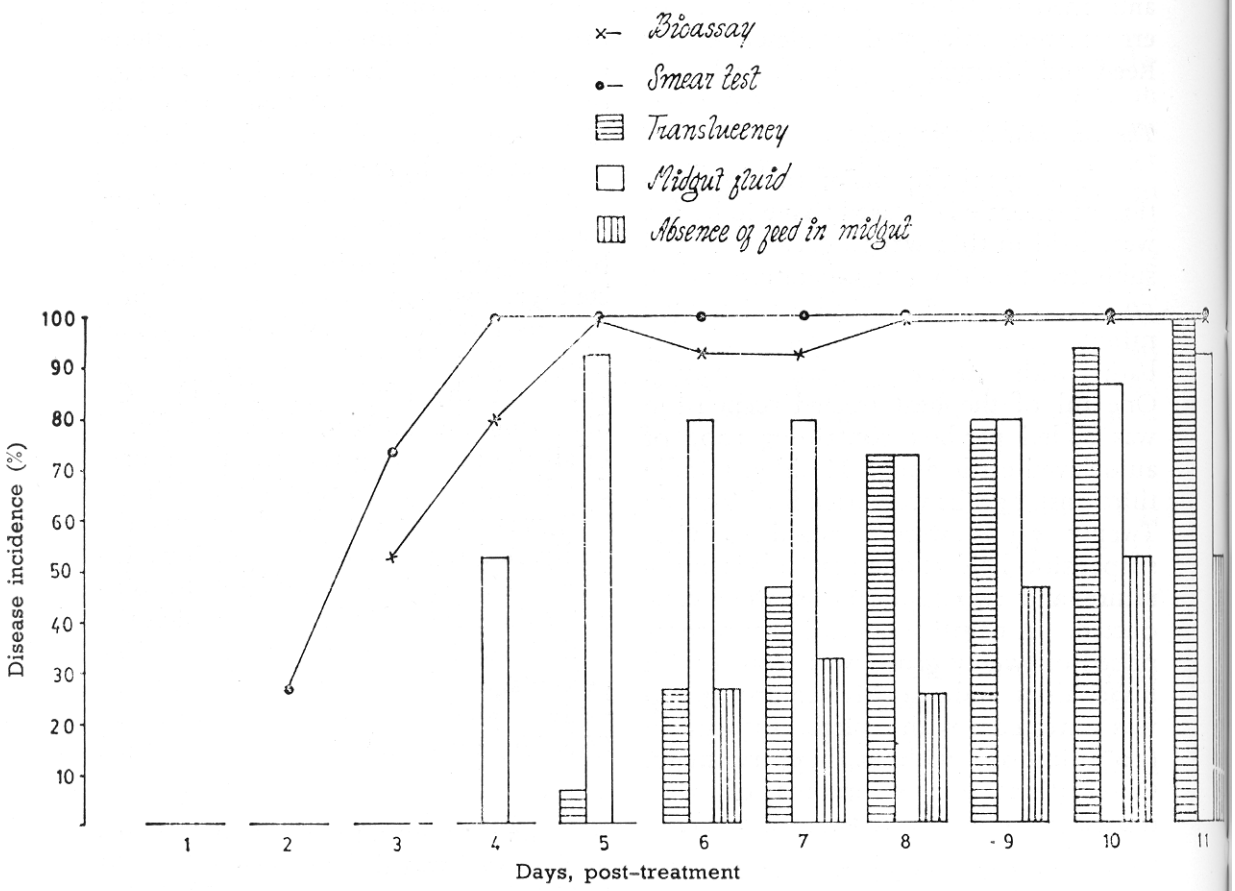
RESULTS AND DISCUSSION

Effect of baculovirus infection on grubs

Fig. 1 depicts the consolidated results of three replications. Each bar represents the presence of a particular symptom (%) among the grubs sampled (15 nos), on a single day. Translucency or waxy appearance of the dorsal side of the thorax was the most striking external symptom of virus-infected grubs. This was due to the absence of feed material in the midgut, since infected grubs ceased feeding and became lethargic. The turbidity of the cuticle was lost and the grubs appeared shrunken. During the acute phases of the disease (2–3 weeks) the grubs surfaced on the feed material and lay motionless in a state of partial paralysis. The external symptoms of *Oryctes* grubs infected with the Malaysian isolate of *Oryctes* baculovirus, as described by Huger (1966) in his first paper, matched the symptoms observed here with the Indian isolate of baculovirus. Translucency in the infected groups appeared from the fourth day of infection onwards in 8.33% of grubs sampled and steadily rose to 100% on the tenth day (Fig. 1). On dissecting the midgut, feed was found in all the infected grubs till the fourth day, but there was no way of ascertaining if it was freshly consumed or was the undigested remains of the previous intake. Hence, it was difficult to pinpoint the stage of infection at which the grubs totally stopped feeding. The percentage of grubs with feed in the midgut gradually declined from the fifth day, reflecting cessation of feeding.

Another important internal symptom noticed was the presence of turbid fluid in the midgut, from the third day post-infection. The fluid contained flakes of epithelial tissue sloughed-off from the midgut lining. This is a consequence of viral multiplication and cellular destruction. The incidence of fluid accumulation rose sharply to 91.66% in grubs sacrificed on the fifth day and reached a plateau thereafter. Typical enveloped bacilliform particles of *Oryctes* baculovirus measuring 210–250 × 80–100 nm were seen in the midgut fluid (Fig. 2).

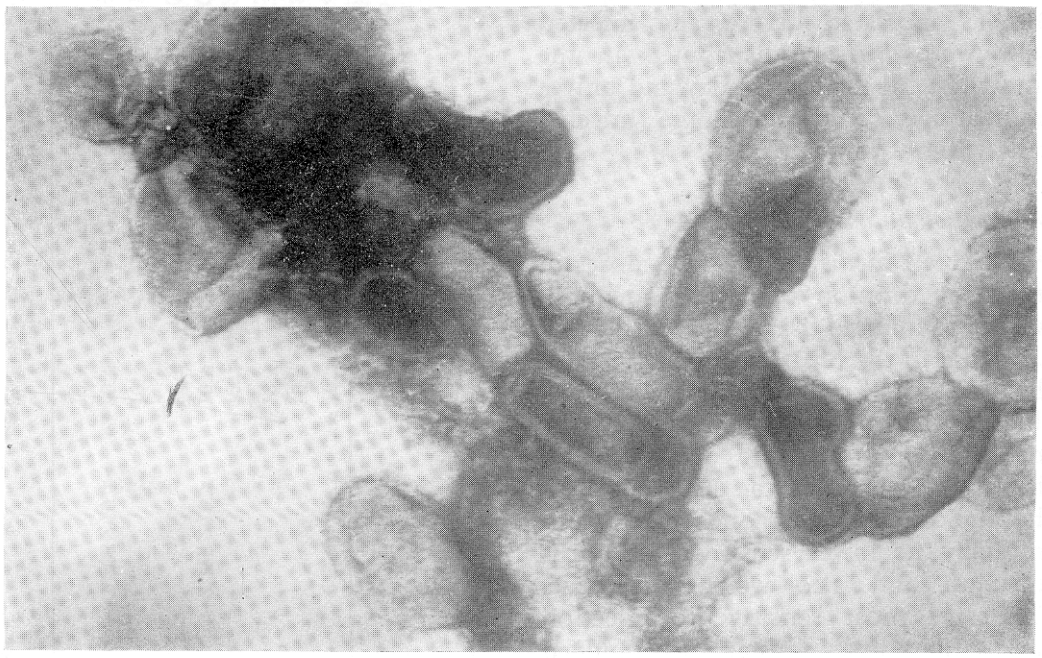
Fig. 1. Sequence of appearance of symptoms and level of detection by the smear and bioassay tests, in virus infected grubs



Grubs in the untreated control group were active and fed normally and no translucency could be perceived even after three weeks. Invariably all the sampled grubs had feed in their midguts and no fluid accumulation at any stage.

Evidence for the multiplication of baculovirus in the midgut epithelium was shown by Giemsa stained smears of midgut epithelial cells. Initial stages of infection could be inferred in 25% of grubs sampled on the first day of post-treatment (Fig. 1). In such smears very few cells possessed the characteristic hypertrophied nuclei (site of viral replication, being a DNA virus) and were sparsely distributed. The fact that infection could be detected as early as 24 hr post-treatment makes the smear test a sensitive diagnostic method for detecting baculovirus disease in grubs. This method is being used widely for the diagnosis of baculovirus disease in

Fig. 2. Electron micrograph of baculovirus infected midgut fluid showing characteristic enveloped virus particles



O. rhinoceros beetles (Zelazny, 1978; Gorick, 1980) and is found to be highly reliable (Mohan et al., 1983). Diagnosis became easier on subsequent days, especially on the fifth and sixth days, because a large number of midgut cells possessed hypertrophied nuclei. Detection of infection in smears in sampled grubs improved to 75% on the third day and 100% on subsequent days. Acutely infected cells with ring stages of the hypertrophied nuclei (Mohan et al., 1983) were frequently seen from the fifth day onwards and gradually outnumbered the other stages towards the end of the experiment *viz.*, 8th–11th day. The nucleus of a typical infected cells measured 20–28μm.

The midgut scrappings of control grubs on all days showed healthy epithelial cells with well defined, considerably small nuclei (8–14 μm) surrounded by distinct cytoplasm. The most distinguishing feature of a normal nucleus was the speckled appearance of the chromatin network.

Bioassay test of the sampled grubs was negative till the second day, indicating that the titer of infective virus particles was below the threshold level of detection in bioassay test. On the third day, 58.3% of the grubs sampled proved to be infective and the incidence reached 100% on the eighth day. Comparing the two methods of

diagnosis, the smear test was able to detect infection even on the second day, when the bioassay recorded negative. But, as indicated before, the infected cells were sparsely distributed in the smear test till the fourth day and only a trained eye could detect the infected cells without difficulty.

Median lethal dose LD_{50}

Table I furnishes the LD_{50} doses in terms of dilution of virus inoculum, weight of infected tissue constituting one LD_{50} dose in the bioassayed grubs. Also the time taken for the appearance of translucency and mortality in each of the log dilution groups are given. In all the three replications the LD_{50} dose was the highest for the second instar grubs implying its lower sensitivity to virus infection and also weight of tissue. The stage most sensitive to infection was the first instar as judged by low LD_{50} and tissue weight containing an infective dose. The difference in LD_{50} between the first and the third instars was only two-fold. The responses did not vary much between replicates for each instar, exception being in the second trial in third-instar. Zelazny (1972) working with the Malaysian isolate of *Oryctes* baculovirus observed very little difference between the LD_{50} concentrations of the different instars inferring homogeneous response, but in the experiment the responses varied considerably between the replicates to the extent of log two.

The infection time was classified into time taken for i) Appearance of translucency (AT) and ii) Lethal Infection period (LIP). In contrast to the

Table I. *Median lethal doses (LD_{50}) and infection period in different larval instars*

Larval instars	Replications I	II	III	Geometric mean of LD_{50}	Dosage	Infection period: Appearance of translucency (days)	Infection period: Lethal infection period (days)
First	2.70×10^{-3}* (SE ± 0.190) 2.760 mg**	1.26×10^{-3} (SE ± 0.254) 6.424 mg	4.79×10^{-3} (SE ± 0.211) 1.410 mg	2.534×10^{-3}	10^{-2} 10^{-3} 10^{-4}	6.72 7.40 No infection	11.45 11.21
Second	2.24×10^{-2} (SE ± 0·255) 34.08 mg	1.86×10^{-2} (SE ± 0.294) 41.04 mg	4.79×10^{-2} (SE ± 0.312) 15.93 mg	2.712×10^{-2}	10^{-2} 10^{-3} 10^{-4}	8.69 8.00 8.00	12.25 13.00 16.00
Third	2.40×10^{-3} (SE ± 0.220) 3.179 mg	2.95×10^{-2} (SE ± 0.251) 25.22 mg	3.16×10^{-3} (SE ± 0.220) 2.192 mg	1.308×10^{-3}	10^{-2} 10^{-3} 10^{-4}	10.03 10.53 No infection	13.10 13.67

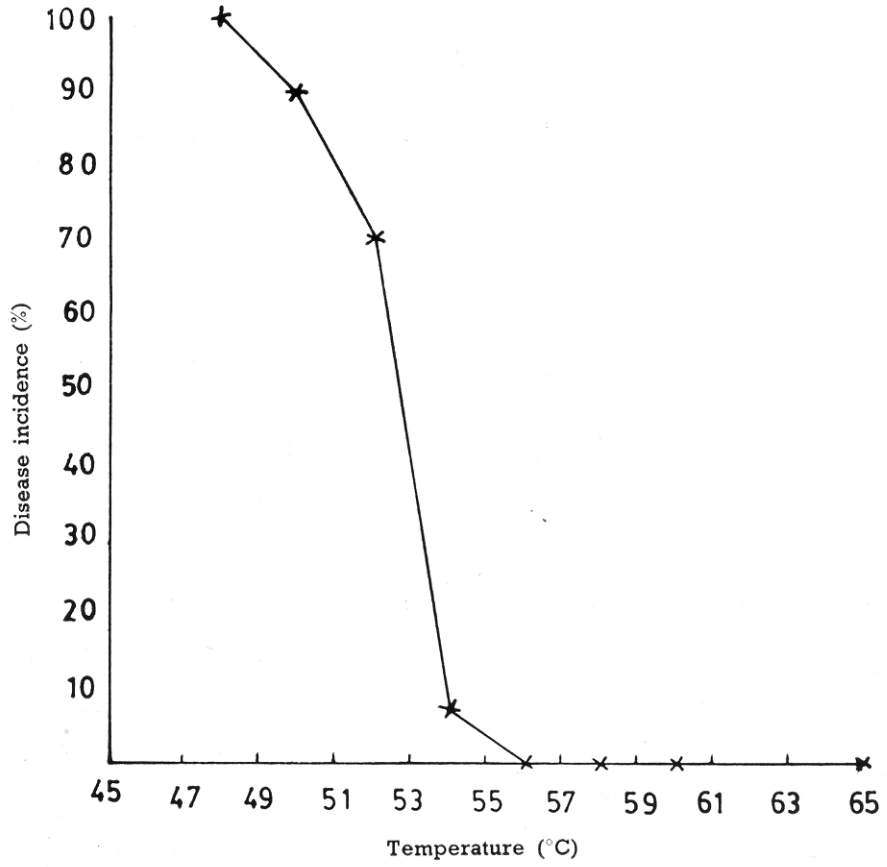
* LD_{50} dose in terms of dilution of virus inoculum
** Weight of infected tissue in mg containing one LD_{50} dose

high LD_{50} values for second instar grubs the periods of translucency and mortality did not vary from those of the third instar. The AT and LIP of first instar were the shortest substantiating the sensitivity to virus infection. On an average, it was found that translucency appeared three to four days prior to death in all the dosages of virus inoculum and there was little difference in the responses between the dosages, implying that increased doses of inoculum do not evoke faster responses in the grubs. Also LIP between instars varied only by a day. In contrast, Zelazny (1972) reported significant variance of LIP between instars, (8.5± 0.2 days for first instar; 12. 6 ± 1.0 day for second instar 22.5 ± 2.8 days for third instar), when fed with baculovirus-killed grubs at 10^{-4} dosage.

Temperature of inactivation

Fig. 3 depicts the heat inactivation profile of *Oryctes* baculovirus. The temperature at which total inactivation of the

Fig. 3. Heat inactivation curve of *Oryctes* baculovirus



virus occurred was found to be 56°C. Zelazny (1972) reported total inactivation at 70°C for 10 min and could detect activity of 15% in viral suspension treated at 60°C for 10 min. The heat inactivation profile could be influenced by the suspension fluid, presence of aggregates of viral particles etc. In this experiment, the virus particles were suspended in the diseased tissue macerate in phosphate buffer, akin to the procedure described for plant viruses. This also suggests the possibility of *Oryctes* baculovirus being subjected to rapid inactivation in the breeding sites of *O. rhinoceros* comprising decomposing organic debris. Nevertheless, the ease with which the disease had spread and established in the pest population in many of the South Pacific Islands leads us to believe that the rate of transmission, vertical and horizontal, outstrips the rate of inactivation.

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