

INFECTIVITY OF *Oryctes* NUDIVIRUS PRODUCED ON CELL CULTURE DSIR HA-1179 AGAINST LARVAE AND ITS EFFECTS ON FEEDING OF NEONATES OF RHINOCEROS BEETLE, *Oryctes rhinoceros*

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ABSTRACT

The *Oryctes nudivir* (OrNV) is a classical biocontrol agent for a major oil palm insect pest the rhinoceros beetle, *Oryctes rhinoceros*. The infectivity of three Malaysian indigenous types of OrNV types A, B and C were tested on larvae and neonates. On larvae, the peroral inoculation test technique indicated that the highest mortality of 100% was achieved using type A produced from cell culture DSIR-HA-1179, while the highest infectivity of 41.7% was recorded for type A prepared from infected guts. No differences in infectivity were observed on other treatments, which ranged from 13.1% to 41.7%. In the substrate contamination inoculation test technique, results showed that the level of infectivity was even lower in all OrNV treatments, ranging as low as 6.7% to only 15.0%. Low infectivity was mainly due to inactivation of virus inocula in the larval food substrates. Based on the results for both inoculation methods, the OrNV type C prepared from cell culture DSIR-HA-1179 was found more effective in controlling the L3 larvae than the other types of OrNV. The impact of OrNV infection on food consumption by the neonates was studied. The feeding of inoculated neonates with OrNV reduced rapidly, especially at the early stage of the experiment between eight days after treatment (DAT) to 16 DAT. At this period, the food consumption by all tested OrNV was rapidly reduced and maintained low until the experiment ended at 60 DAT. The highest feeding reduction rate was on neonates treated by type A (-0.074x) followed by neonates treated by type C (-0.053x) and type B (-0.035x). Therefore, it was suggested that besides on highly virulent, the selection of OrNV for field release should also based on high reduction rate on food consumption by the infected insects on plant hosts.

Keywords: *Oryctes rhinoceros*, *Oryctes nudivir*, oil palm insect pest, food consumption.

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INTRODUCTION

The outbreaks of economically important pests, such as the rhinoceros beetle, *Oryctes rhinoceros* have greatly affected the sustainability of the oil palm development in Malaysia. Reasons that contribute to the outbreak are highly associated with the abundance of breeding sites due to prohibition of open burning in the plantations during the early stage of land preparation (Bedford, 2013a). The

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attacks of rhinoceros beetle on oil palm occurred in both mature and immature areas (Norman and Basri, 2004). However, the attack is more lethal to the young growing palm below 4 years old (Cik Mohd Rizuan *et al.*, 2014).

Feeding activity by the adults causes the formation of wedged-shaped leaves when the young fronds unfold and this affects the yield of the palm (Samsudin *et al.*, 1993). The entry feeding holes left by the rhinoceros beetles could attract a secondary pest such as *Rhynchophorus ferrugineus* (Wood, 1968). The photosynthetic areas of the severely attacked palm can be reduced up to 15%, thus, delaying the palm maturity as well as reducing the yield of the palm (Samsudin *et al.*, 1993). Chung *et al.* (1999) estimated that losses due to the infestation of beetles on 21-month old palm has lowered the production of fresh fruit bunches (FFB) to almost 80% in the first year after attack.

The arthropod-specific entomopathogen virus, the *Oryctes* nudivirus (OrNV) is one of the natural enemies to hamper the beetle populations in the plantations. The virus was isolated from larvae in Malaysia (Huger, 1966), and has been proven to be lethal to larvae and adults of rhinoceros beetles (Huger, 2005). Larvae get infected via feeding on the virus contaminated substrates, while infection on adults was mostly via mating (Zelazny and Alfiler, 1991). The adults were found acting as a virus reservoir, transmitting the virus within the population. The virus then replicated in the gut tissues, thus reducing the feeding activity as well as the fertility of females (Zelazny, 1973). Following a successful pilot release in Samoa, which significantly reduced the beetle population, the OrNV was then introduced in many South Pacific and Indian Ocean countries (Marschall, 1970; Zelazny and Alfiler, 1991). In Fiji, the impact of OrNV helped to suppress and maintained the beetle population below the economic threshold level even up to 40 years after the introduction (Bedford, 2013b).

The cell line DSIR-HA-1179 was established from the African black beetle *Heteronychus arator* (Crawford, 1982) and it was the only cell line that supports the *in vitro* propagation of the OrNV (Crawford and Sheehan, 1985). As this technology enables the production of OrNV with high purity, it has been widely used by various researchers to release the OrNV to control rhinoceros beetles in both coconut and oil palm plantations (Crawford and Sheehan, 1984; Zelazny *et al.*, 1987). Our previous study showed that the Malaysian OrNV types A, B, and C have been successfully produced on insect cell line DSIR-HA-1179 and all OrNV inocula were proven highly pathogenic on adults rhinoceros beetle.

This research further evaluated the infectivity of the cell-propagated OrNV and the wild-collected OrNV on the larvae of the rhinoceros beetles using

peroral and substrate contamination inoculation methods. The feeding behaviours of neonates of rhinoceros beetle inoculated with the OrNV were also studied.

MATERIALS AND METHODS

Source of OrNV Solution

All the three OrNV solutions, types A, B and C were prepared from two weeks old insect cell line DSIR-HA-1179, provided by the AgResearch, New Zealand. The cells were grown in a 25 cm² flask with 5 ml PS100 medium containing 10% fetal bovine serum (FBS) and antibiotics. Propagation of all types of OrNV in cell cultures was performed following the method of Farhah *et al.* (2015). The initial seeding density used in each flask was at 1×10^5 viable cells/ml. A total of 100 μ l of OrNV solution prepared from homogenated infected gut tissues of adults of rhinoceros beetle from respective localities were added into the cell culture. The OrNV type A solution was prepared from the infected guts of adults collected from Munkar Estate in Johor, Malaysia, type B from Seberang Perak Estate in Perak, Malaysia and type C from Lahad Datu, Sabah, Malaysia. Prior to inoculation, the respective OrNV solutions were purified by double filtration through; first a 0.45 μ m, and second a 0.22 μ m membrane filter to avoid the presence of other microorganisms.

Source of Larvae and Neonates

The third instar larvae (L3) were collected from breeding sites at Regent Estate, IOI in Melaka, Malaysia. The L3 larvae were determined by measuring the diameter of the head capsules with an average of 9.1 mm (Norman, 2001). Larvae with any signs of viral or fungal infection were discarded and only the healthy larvae were picked up and placed into a plastic container with dimension of 60 cm long \times 45 cm wide \times 30 cm high, half filled with rotting chipped palm trunks. The collected larvae were brought back to an outdoor insectory for rearing. The larvae were then kept individually in a 500 ml plastic cup filled with moist rotting chipped oil palm trunks. The temperature of the insectory was maintained at 25°C to 28°C. The food source in each cup was replaced every two weeks until the larvae turned to neonates.

DNA Extraction for PCR Analysis

The DNA of OrNV was extracted from the infected gut of both larvae and adults following the protocol as described by Ramle *et al.* (2010). The gut tissues were macerated and then briefly

centrifuged to pellet the gut tissues. A total of 150 μl of solution were transferred into a new 1.5 ml microtube. A volume of 300 μl disruption buffer containing 50 μl 1M Tris pH8, 10 μl 0.5M EDTA, 5 μl 10 % SDS, 2.5 μl proteinase K (20 mg ml⁻¹), and 232.5 μl sterilised milliquate water (SMqH₂O) was added into the tube. The mixture was then shaken and incubated at 65°C for 60 min. Then, equal amount of phenol-chloroform-isoamylalcohol (PCI) (25:24:1) was added into the microtube. The mixture solution was mixed by inversion for several times. After centrifugation for 10 min at 13 000 rpm, 450 μl of the upper solution were pipetted and transferred into new microtube. Precipitation of OrNV DNA was then conducted by filling 10% NaAc pH 5.2 and two times volume of 100% ethanol into the microtube. The mixture was then kept at -20°C for an hour. The OrNV DNA was pelleted by centrifugation at 13 000 rpm for 10 min, then rinsed twice by adding 100% alcohol, air-dried and finally suspended in 100 μl TE buffer.

Polymerase Chain Reaction (PCR) Analysis for Detection of OrNV

The PCR analysis conducted in this study followed the optimised techniques by Ramle *et al.* (2010), using a pair of primers 15a (5' - ATT ACT TCG TAG AGG CAA TC - 3') and 15b (5' - CAT GAT CGA TTC GTC TGT GG - 3') developed by Richards *et al.* (1999). The PCR was carried out in 0.2 ml microtube by mixing 4.0 μl of OrNV DNA and 22.0 μl lysis buffer containing 14.63 μl of SMqH₂O, 1.25 μl of MgCl₂, 2.5 μl of 10X PCR buffer 10 mM, 0.5 μl DNTP, 1 μl of each primer 15a and 15b, 0.125 μl Taq DNA polymerase, and 1 μl bovine serum albumin (BSA) (20 mg ml⁻¹). The addition of BSA was used to improve the yields of DNA in the PCR (Farell and Alexandre, 2012). Samples used for positive and negative control were included in each reaction. Sample for positive control used the previously confirmed OrNV DNA, whereas sample for the negative control used only the SMqH₂O. The PCR was performed in a thermal cycler machine (Vapo Protect, Eppendorf) run for 30 cycles; denaturing at 94°C for 1 min, annealing at 72°C for 2 min and extension at 50°C for 1 min. The PCR products were electrophoresed on 1.8% agarose gel prepared using 1X TAE buffer with ethidium bromide. The gel was then visualised under the UV transillumination. The presence of single DNA band at 945 bp confirmed that the sample was infected by OrNV.

Experiment 1: Infectivity of cells-propagated and field-collected OrNV on larvae tested by peroral inoculation technique. Three types (type A, B, and C) of OrNV from two different preparations of inoculum were tested against the early stage of L3 larvae of rhinoceros beetle. First inoculum of OrNV

was prepared from two weeks old insect cell culture produced following the method of Farhah *et al.* (2015). The second inoculum was prepared from the infected gut tissues of adults collected from estates in Johor for type A, in Perak for type B, and in Sabah for type C. The study was carried out in a randomised complete block design (RCBD) with three replicates for each treatment. The number of tested larvae in each replicate was five larvae, accounting for a total of 105 larvae (90 larvae used in treatments and 15 larvae in control). Larvae were collected from the same locality and maintained as previously described. Prior to bioassay, the infection level of OrNV on larvae was estimated using PCR analysis from 10% of total larvae used in the study. The PCR results showed that the samples of larvae were free from OrNV infection.

Prior to inoculation of larvae, the concentrations of each type of OrNV prepared from both inocula were estimated by PCR-comparative method and then standardised at 1-2 $\times 10^4$ infectious unit (IU) ml⁻¹ in 10% sucrose solution. Inoculation of larvae was conducted peroral by placing 30 μl of OrNV solution onto the mouthparts of the larvae. A group of five inoculated larvae were placed in a plastic container with dimension of 11 cm width \times 17 cm length \times 7.5 cm height, filled with chipped rotten oil palm trunks as food source. For control, the larvae were only inoculated with 30 μl of 10% sucrose solution. Mortality of the larvae was recorded every four days after treatment (DAT) until one of the treatments achieved 100% mortality. The dead larvae were dissected and the midgut tissues were taken for confirmation of OrNV infection using the PCR method as described above.

Experiment 2. Infectivity of cells-propagated OrNV on larvae tested using substrate contamination inoculation technique. This experiment only tested OrNV types A, B, and C propagated on cell culture DSIR-HA-1179 against the late stage L3 larvae using substrate contamination inoculation technique. The age of larvae was about 30 days older than the larvae used in Experiment 1. The preparation and standardisation of OrNV inocula were conducted as in Experiment 1. A total of 10 ml of OrNV solutions prepared in 10% sucrose were directly sprayed onto the larval breeding substrates which comprised of chipped rotten oil palm trunks in a container. Before adding a group of five larvae into the container, the substrates were homogenously mixed. In control, the breeding substrates were left uninoculated. The containers were then placed in dark condition at 25°C to 28°C to minimise the effects of biotic factors in degrading the OrNV inocula. The mortality of larvae was inspected every four DAT, until one of the treatments reached 100% mortality. All the dead larvae were subjected to PCR analysis to confirm the OrNV infection.

Experiment 3. Effects of OrNV infectivity on feeding behaviour of neonates of rhinoceros beetle.

The OrNV types A, B, and C propagated from cell culture were used in this study (Farhah *et al.*, 2015). The concentration of each type of OrNV was set at $1-2 \times 10^4$ IU ml⁻¹. The study was carried out in a RCBD with five replicates per treatment, consisted of five neonates for every replicate. Neonates of rhinoceros beetle were obtained from rearing of L3 larvae as previously described. A day before the administration of OrNV inocula, the neonates were placed inside an empty container for fasting overnight, as this will facilitate the inoculation of OrNV to neonates (Ramle *et al.*, 2011). Administration of OrNV was conducted by placing 30 ul of virus inoculum onto the mouthparts of the neonates (Ramle *et al.*, 2011). The inoculated neonates were kept in a transparent plastic container and fed with several pieces of sugar-cane of about 20 cm long. The infectivity of OrNV and food consumption of neonates was recorded every four days until 60 DAT. All the dead neonates were subjected to PCR analysis to confirm the OrNV infection following the method of Ramle *et al.* (2010).

Statistical Analysis

The cumulative percentage of mortality and infection was firstly corrected using the Abbott's Formula (Abbott, 1987) then angularly transformed before analysis of ANOVA was performed. The means were separated using Duncan's Multiple Range Test at P=0.05 (SAS, 1997). The mean of food consumption was subjected to ANOVA (SAS, 1997). The rates of food consumption over time were plotted using a negative exponential curve graphs by SigmaPlot 10.0.

RESULTS AND DISCUSSION

Infectivity of Cells-propagated and Field-collected OrNV on Larvae Tested by Peroral Inoculation Technique

All types of OrNV prepared from both inocula, cell culture or gut tissues, were capable to cause mortality on larvae of rhinoceros beetle (Table 1). The mortality of larvae treated by all types of OrNV increased at slower rate starting from 4 DAT and reached more than 50% at 20 DAT, then peaked at the highest level at 30 DAT. During this period, the OrNV type A inoculum prepared from cell culture recorded the highest mortality, starting from 20 DAT up to 30 DAT. This trend on high mortality on larvae inoculated with OrNV prepared from cell culture was clearly observed again on the OrNV type C as early as 12 DAT, where the treatment CC killed 21.7%, while no mortality was recorded for treatment CG (Table 1). Similar trend was recorded again on the OrNV type C and type A at 16 DAT. Starting from 20 DAT up to 30 DAT, the mortality of larvae treated with all types of OrNV prepared from cell culture was always higher as compared to larval mortality caused by the OrNV from the infected gut tissues. The OrNV type A from cell culture (AC) recorded the highest mortality of 100%, followed by the treatments BC (88.9%), BG (77.8%), CC (77.8%), AG (72.2 %), and CG (61.1%).

The findings showed that type A of OrNV was capable of killing 100% larvae at 30 DAT. The result was in agreement with that of Gopal *et al.* (2001), who found that the infected larvae took less than a month to die. Finding in this study was contrary with the study by Ramle *et al.* (2011), who found that the highest mortality was caused by the OrNV type

TABLE 1. PERCENTAGE MORTALITY AND INFECTIVITY OF LARVAE AFTER TREATED WITH *Oryctes* NUDIVIRUS TYPES A, B AND C PREPARED FROM CELL CULTURES AND INFECTED GUT TISSUES USING PERORAL INOCULATION TECHNIQUE

Type of OrNV	Source of inoculum (acronym)	Cumulative mortality (%) at day after treatment (DAT)								Total infectivity* (%)
		4	8	12	16	20	24	28	30	
Type A	Gut tissues (AG)	6.7 a	13.3 a	20.0 a	38.3 ab	41.7 b	50.0 b	69.4 a	72.2 a	41.7 a
	Cell culture (AC)	0.0 a	0.0 a	12.2 a	48.9 a	83.3 a	91.7 a	100.0 a	100.0 a	13.1 b
Type B	Gut tissue (BG)	6.7 a	13.3 a	20.0 a	30.0 ab	36.1 b	47.2 b	63.9 a	77.8 a	13.1 b
	Cell culture (BC)	6.7 a	6.7 a	13.3 a	21.7 b	44.4 b	63.9 ab	72.2 a	88.9 a	13.1 b
Type C	Gut tissues (CG)	0.0 a	0.0 a	0.0 a	21.7 b	38.9 b	47.2 b	61.1 a	61.1 a	28.1 ab
	Cell culture (CC)	0.0 a	0.0 a	21.7 a	30.0 ab	44.4 b	52.8 b	77.8 a	77.8 a	35.0 a

Note: * Percentage of infectivity was calculated based on cumulative data recorded at 30 DAT.

Means in columns with the same letters were not significant different by Duncan's Multiple Range Test (DMRT) at P = 0.05.

B (86.7%) followed by type A (26.7%) and type C (13.3%) on larvae treated with OrNV prepared from infected guts.

Apart from the virus infection, high larval mortality could presumably be due to infection by other microbes such as bacteria and flagellates. Norman *et al.* (2007) reported that infection by *Bacillus thuringiensis* and *B. popillae* had caused mortality to larvae ranging between 25% to 64%. The infected larvae turned pulpy and became black-brownish or bluish in colour. The infection of *Acinetobacter calcoaceticus* and flagellum species *Monocercomonoides oryctesae* and *M. qadrii* on larvae were also reported (Krishnamurthy and Sultana, 1977; Kannan *et al.*, 1980). Another factor contributing to high larval mortality was possibly due to competition from other microorganisms in the gut lumen, as the larval gut content is rich in organic matters and suitable for the growth of microorganisms (Bedford, 2013a).

It was clearly shown in this study that despite of having higher mortality, most of the larvae were dead not due to the OrNV infection (Table 1). The percentages of dead larvae infected with OrNV ranged from 13.1% and 41.7%. The infectivity of OrNV prepared from different sources of inoculum seems to be inconsistent. Among treatments, the AC, BG, and BC have the same and yet the lowest infectivity of 13.1%. The highest infectivity was recorded in treatment AG at 41.7%, followed by the treatments CC at 35.0% and CG at 28.1%. Based on the percentage of the total infectivity, the results in this study contradicted to that of Ramle *et al.* (2011). In this experiment, the type C prepared from both sources of inoculum had high infectivity on the larvae as compared to the type B in the latter study done by Ramle *et al.* (2011).

In the natural breeding sites, among the larval instars, the L3 larvae were the most affected by the infection of OrNV (Ramle *et al.*, 2011). The OrNV infection on the third instar larvae ranging from 29% to 35%, followed by the second instar (0% to 25%) and lastly the first instar larvae (0% to 3%). This was mainly due to the fact that L3 had the longest lifespan of 112.5 days as compared to the other instars (Bedford, 1980). This explains why it is unavoidable to get samples free from infection, especially larval samples in the control. Although pre-assessment analysis by PCR before the experiment showed that none of the larvae were infected, but some of them get infected 13.3% at the end of the experiment. This could possibly be due to low numbers of larvae used in the pre-assessment analysis or frequent replacement of new breeding substrates. These substrates could contain OrNV particles as these materials were collected from field and not sterilised.

This also explains the low infectivity rates by OrNV on the larvae. As the larvae used in this study

were L3 collected from the plantations, they have been exposed to other microbes from the natural food sources and the environment. The microbial activity within the guts may have interfered with the virus infection in larvae (Ramle *et al.*, 2011; Bedford, 2013a).

No significant difference on total infectivity was recorded between the larvae inoculated with OrNV prepared from infected gut tissues and from cell cultures, except for type A. The infectivity results of all OrNV prepared from cell culture in this study supported the earlier findings by Zelazny *et al.* (1987) who found that OrNV produced from cell culture could induce high infectivity (Table 1). Thus, these findings could provide an insight that the OrNV produced on cell culture has a potential to be used to control the larvae of rhinoceros beetle in the field, provided that a more economical way to produce the virus is readily available.

Infectivity of OrNV Propagated on Cell Culture on Larvae Tested by Substrate Contamination Inoculation Technique

In this study, all types of OrNV were produced from two weeks old cell cultures. The larvae were inoculated by substrate contamination technique using large volume of virus inoculum, about 10 ml per container. As the L3 larvae used in this experiment were about 30 days older than the L3 larvae used in Experiment 1, therefore, in most of the treatments, some of the larvae had turned to pre-pupae and pupae. These new pupae were proved to be free from OrNV infection by PCR analysis and previous research also stated that none of those pupae collected from the fields were infected by OrNV (Ramle *et al.*, 2011).

The larval mortality was gradually increased starting from between 0.0% and 6.7% at four DAT to between 50.0% and 66.7% at 30 DAT (Table 2). From four DAT to 28 DAT, the larval mortality treated by OrNV type B was always slightly higher than the other treatments (Table 2). At 30 DAT, OrNV types B and C recorded the highest mortality of 66.7% followed by type A of 50.0%. Based on the total infectivity, the OrNV type C recorded the highest infection of 15.0%, followed by OrNV types A and B of only 6.7%. Surprisingly, the high infection of OrNV type C on larvae in this Experiment 2 was also recorded in Experiment 1, where the type C prepared from cell culture had 35.0% infection compared to infection by types A and B (13.1%) (Table 1).

As in Experiment 1, higher mortality and even lower infectivity of OrNV on larvae was again recorded in this experiment. Possible explanations for the high larval mortality have been elaborated in Experiment 1. As expected, the infectivity of OrNV on larvae was even lower as compared to

TABLE 2. PERCENTAGE MORTALITY AND INFECTIVITY OF LARVAE AFTER TREATED WITH *Oryctes* NUDIVIRUS TYPES A, B AND C PREPARED FROM CELL CULTURES USING SUBSTRATE CONTAMINATION INOCULATION TECHNIQUE

Type of OrNV	Corrected cumulative mortality (%) at day after treatment (DAT)*								Total infectivity (%)*
	4	8	12	16	20	24	28	30	
Type A	0.0 a	0.0 a	6.7 ab	15.0 a	23.3 a	38.9 a	50.0 a	50.0 a	6.7 a
Type B	6.7 a	13.3 a	26.7 a	35.0 a	45.0 a	50.0 a	66.7 a	66.7 a	6.7 a
Type C	0.0 a	0.0 a	0.0 b	20.0 a	20.0 a	25.0 a	58.3 a	66.7 a	15.0 a

Note: *Percentage of infectivity was calculated based on cumulative data recorded at 30 DAT.

Means in columns with the same letters were not significant different by Duncan's Multiple Range Test (DMRT) at $P = 0.05$.

There were five individuals in each replicates which made up a total of 15 individuals for each treatments including control.

level of infection recorded in Experiment 1. This is because in this experiment, inoculation was made by spreading the OrNV solutions onto breeding substrates, thus exposing the OrNV non-enveloped virion particles to the detrimental abiotic factors. Inactivation of OrNV has been reported due to unfavourable environmental factors such as high temperature, low moisture and microbial activity in breeding materials of the beetles (Wang *et al.*, 2012; Bedford, 2013a). Paulose and Abraham (2002) also found that the OrNV solutions made up from the macerated infected L3 larvae were completely deteriorated after being exposed for more than 4 hr at 37°C. Bedford (1980) claimed that the virus particles might quickly disintegrate when they were mixed with the larva-rearing substrates. Furthermore, Bedford (2013a) added that a more virulent OrNV would cause rapid death to the insects, thus deteriorated the living host of the virus and resulted in rapid inactivation of the OrNV in the environment.

The evidence of low infectivity of substrate contamination inoculation as compared to peroral

inoculation techniques in this study was also reported by other previous researchers. These included reports by Zelazny (1978), Zelazny and Alfiler (1986), and Zelazny *et al.* (1987), which discovered that the peroral inoculation of OrNV caused much higher infection on the rhinoceros beetle. Jayawardena (2013) has also reported that a significantly higher infection by OrNV was recorded by peroral inoculation as compared to swim inoculation method.

Effects of OrNV Infectivity on Feeding Behaviour of Neonates of Rhinoceros Beetle

The reduction in food consumption by neonates of rhinoceros beetle inoculated with cells-propagated OrNV types A, B and C is shown in Figure 1. Although, the food source used in this study was sugar-cane as replacement for the actual food source of the beetle, which is the oil palm tissues, the results can be used to estimate the feeding behaviours of the adults. As early as four DAT, the inoculated neonates fed at almost similar rate with those in control, except for

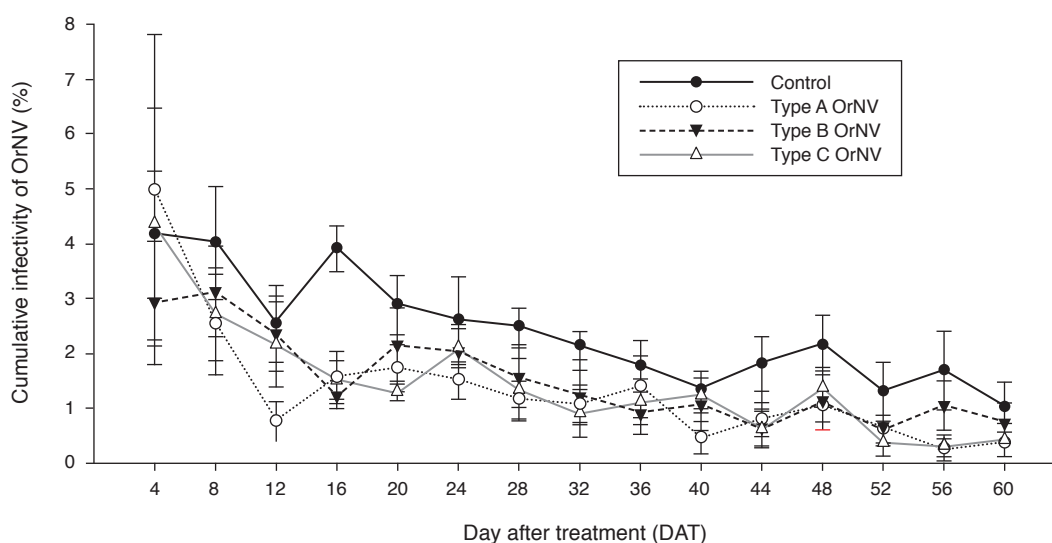


Figure 1. Effects of *Oryctes nudivirius* (OrNV) types A, B and C in reducing the food consumption by the inoculated neonates of *Oryctes* rhinoceros.

neonates treated with the OrNV type B. At this stage, replications of the inoculated virus in the gut tissues have just begun and did not affect the feeding of the neonates. Interestingly, starting from eight DAT to 16 DAT, rapid reduction in food consumption in all treatments was obviously observed, particularly for the OrNV type A. Food consumption by neonates inoculated with the OrNV type A was significantly reduced ($P < 0.05$) from 3.3 g at eight DAT to 0.9 g at 12 DAT. Rapid decrease in food consumption for types B and C was started at 16 DAT from about 3.3 g to 3.5 g at eight DAT, then reduced to 1.5 g at 16 DAT. At 16 DAT onwards, the food consumption by all treated neonates were reduced, but at a more slower rate.

Substantial reduction in food consumption was perhaps due to the massive replication of OrNV in the midgut tissues of the neonates. Extensive replication of OrNV caused the midgut full with the virus particles which substantially disrupted the natural feeding behaviour of the neonates (Huger, 1966; Huger and Krieg, 1991). Inglis *et al.* (2001) has reported that the impact of OrNV on feeding behaviour of the infected hosts can only be seen between the late stages of infection and before death. This situation was unlikely to happen in the current study. At the early stage between eight DAT to 16 DAT, significant impact of OrNV in reducing the food consumption of neonates was clearly observed. At this period, the PCR analysis on dead neonates found only 5% to 15% of them have died due to infection (Figure 2). Even at 20 DAT, the dead neonates with virus infection was still below 20%. It was suspected that the infection level on live inoculated neonates could possibly be much higher. As the experiment ended at 60 DAT, the infection of OrNV type B was recorded at 88.0%, type A at 68.0% and type C at 56.0%.

The feeding activity by the adults of rhinoceros beetles caused damage to oil palm and reducing yield (Manjeri *et al.*, 2014). The OrNV infection affects the feeding behaviour, as well as the fecundity of the insect hosts as observed in fields by Zelazny (1977). It was obvious that the food consumption in all treatments decreased proportionally with the increased in the infection level by OrNV (Figures 1 and 2). The feeding of the treated neonates decreased over time to below 0.8 g at 60 DAT and always lower than the amount of food taken by the untreated neonates in the control (Figure 1).

Based on rates of food consumed by neonates in this experiment, it has the same trend of negative exponential curves but with the difference in reduction rates depending on treatments (Figure 3). The OrNV Type A has the highest reduction rate of $-0.074x$, then followed by the type C of $-0.053x$, type B of $-0.035x$ and control of $-0.029x$. From the graphs, it revealed that the reduction on food consumption by neonates was constantly reduced at the slower rate. In actual field conditions, those infected neonates were still capable of causing damage to the palm. Therefore, this explains why infestation of rhinoceros beetle in fields is still occurring despite of high infection of OrNV on adults (Ramle *et al.*, 2011). Low effect of virus infection on feeding activity of the neonates was probably due to the adaptation of the beetle immune system against the OrNV. Zelazny *et al.* (1989) have reported that the population of rhinoceros beetle in the Philippines has started showing resistance toward OrNV as compared to beetles in West Samoa. Nevertheless, the advantage of slow feeding of the infected beetles is that it increases chances for the beetles in disseminating the virus to other healthy beetles and later reduces the pest populations in the outbreak areas (Zelazny, 1976).

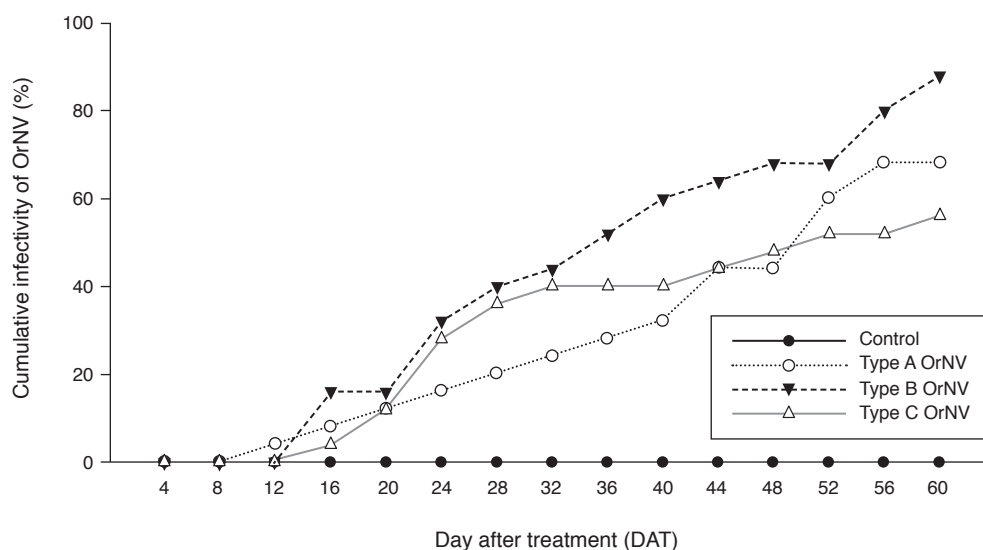


Figure 2. The percentage of cumulative infection of neonates of *Oryctes rhinoceros* inoculated with *Oryctes nudivirius* (OrNV) types A, B, and C.

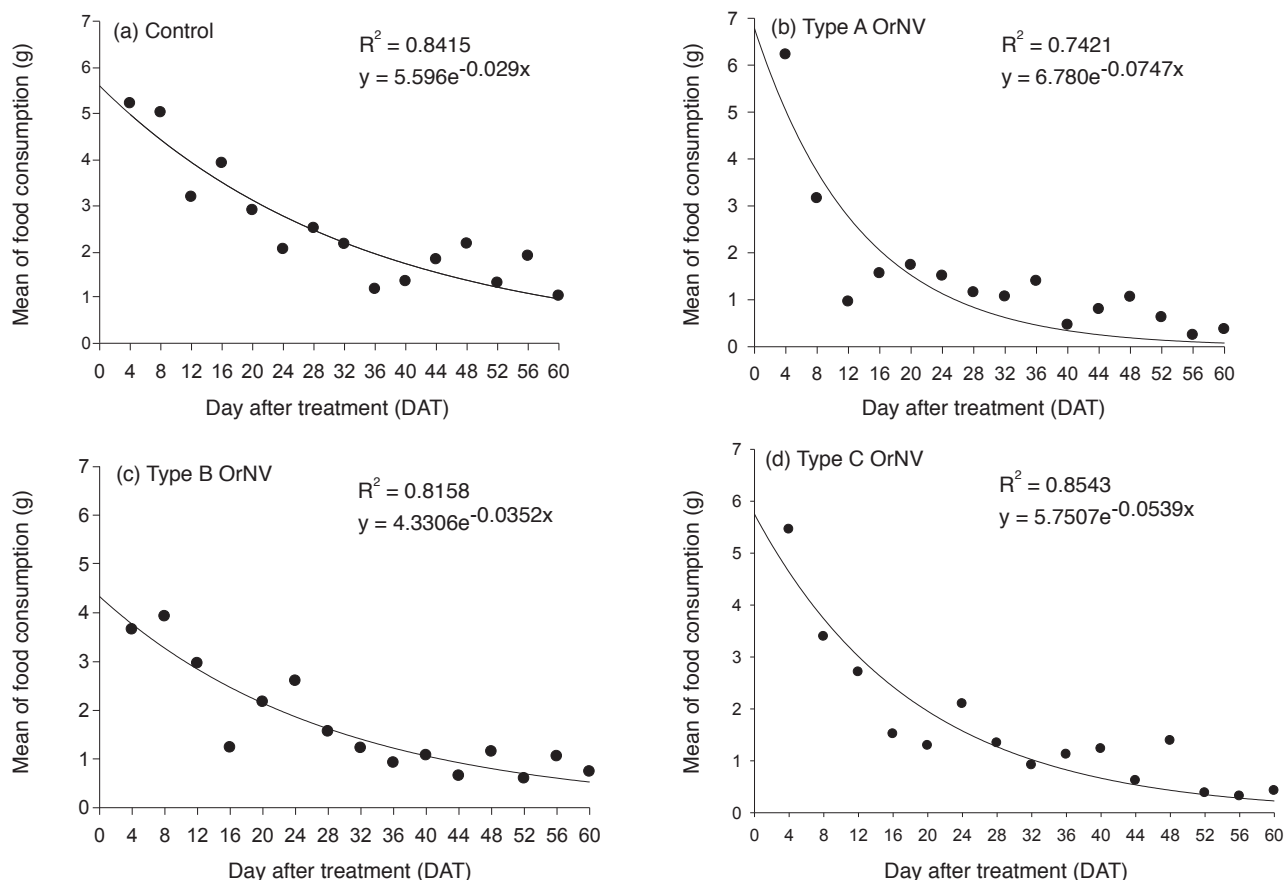


Figure 3. The rate of reduction on food consumption by the neonates of *Oryctes rhinoceros* after inoculated with the *Oryctes nudivirus* (OrNV) types A, B, and C.

CONCLUSION

In summary, this study found that OrNV prepared from the cell culture has comparatively high infectivity as compared to OrNV freshly prepared from the infected gut tissues. On larvae, the peroral inoculation method caused higher infectivity level ranging from 13.1% to as high as 41.7% as compared to food contamination method which caused only between 6.7% to 15.0%. Based on results in both inoculation methods, the OrNV type C prepared from cell culture DSIR-HA-1179 was more effective in controlling the L3 larvae than the other types. However, applying OrNV solutions targeting to control larvae in the breeding sites could expose the virus solutions to various detrimental abiotic factors leading to virus inactivation. The inoculation of OrNV has greatly affected the feeding behaviour of the neonates. The effects were obviously showed at the early stage of the experiment between eight DAT to 16 DAT. During this period, the food consumption by all tested OrNV was rapidly reduced and maintained low till the experiment ended at 60 DAT. Based on feeding reduction rates, type A was found to be the most effective in reducing the feeding of neonates, then followed by type C and type B. This

experiment suggested that selection of OrNV for field release not only solely based on the capability of the virus to kill the insect, but also to account the effects of the virus in reducing the feeding of the insect on the plant host.

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