

MOLECULAR APPROACHES IN THE ASSESSMENT OF *Oryctes rhinoceros* VIRUS FOR THE CONTROL OF RHINOCEROS BEETLE IN OIL PALM PLANTATIONS

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ABSTRACT

The successful introduction of the *Oryctes rhinoceros* virus to control an outbreak of the rhinoceros beetle, *O. rhinoceros* on coconut in the South Pacific islands has led MPOB to embark on a project to study the potential use of the virus in oil palm plantations in Malaysia. Two DNA-based technologies, the polymerase chain reaction (PCR) and the restriction virus genome by an endonuclease enzyme, have been developed and intensively used in the project. PCR is a sensitive yet simple procedure allowing a more accurate estimation of the infection level of the *O. rhinoceros* virus on adult beetles and larvae. PCR diagnosis showed that the adult beetles were more commonly infected (30%-65%) as compared with larvae (0%-35%). Pre-pupae and pupae were free from the virus infection. Virus genomic analysis by an endonuclease enzyme HindIII identified four types of *O. rhinoceros* virus, named as type A, B, C and D. Bioassays showed that the virus type B was more pathogenic against the third instar larvae and neonates. A virus field introduction system was then established. The virus solution was produced by an in vivo method using the larvae and adult beetles. Introduction of the *O. rhinoceros* virus type B in an estate with palms less than one year old with existing virus of type A resulted in a successful reduction of the adult population as well as in palm damage. The released virus established as early as three months after release (MAR) and persisted up to 15 MAR. The virus type B was then introduced in an immature area with palms more than three years old. The virus infection gradually increased and was maintained at a higher level of between 60% and 90%. The adult population was reduced, and stayed at a low level for a certain period of time before slowly increasing again to reach a second peak. The virus infection had a weak negative correlation with the adult population. A slow reduction in the proportion of males was observed, possibly due to slow virus transmission, as the adult population had probably already adapted to the virus infection. Genomic analysis showed that the virus type B was detected only at four MAR. Factors ensuring the success of virus transmission in the population were elaborated upon. Further research to fully utilise the *O. rhinoceros* virus to ensure maximum control of the rhinoceros beetle was also discussed.

Keywords: *Oryctes rhinoceros* virus, pest management, biological control, oil palm insect pests.

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INTRODUCTION

The history of the fascinating discovery of the *Oryctes rhinoceros* virus has been reported by Huger (2005). The devastating damage on coconut caused by the rhinoceros beetle, *Oryctes rhinoceros*, in the South Pacific between the 1950s and 1960s intensified the activities in searching for possible

pathogens of the beetle. Extensive surveys around the tropical belt had identified numerous diseases, but none of them were pathogenic against the beetle (Surany, 1960). In 1963, the search was concentrated in Southeast Asia, especially in Malaysia. The first batch of larvae of the rhinoceros beetle bearing striking infection symptoms of the *O. rhinoceros* virus was discovered. Basic research in identification showed that the disease was caused by a rod-shaped non-occluded insect virus described as *Rhabdionvirus oryctes* (Huger, 1966). Later, the virus was classified in a new genus under the Baculoviridae, Sub-group C, and was commonly cited as *Baculovirus oryctes*. In 1997, the virus was assigned to a new *Oryctes* virus family (Evans and Shapiro, 1997). Recently, it was proposed that the *O. rhinoceros* virus be assigned to a newly established genus Nudivirus, together with the *Heliothis zae* virus 1 (HzV-1) (Wang *et al.*, 2007).

The first release of the *O. rhinoceros* virus in West Samoa in 1970 ended with successful results (Marschall, 1970). Effects of the virus on the susceptibility of larvae and adults, life-span, fecundity of females and mortality of the rhinoceros beetle were then intensively investigated (Zelazny, 1972; 1973; Monty, 1974). Results show that the *O. rhinoceros* virus successfully reduced the beetle population and coconut palm damage. Therefore, the virus release projects were then extended to many coconut-producing countries in the South Pacific islands (Hammes, 1978; Gorick, 1980; Young and Longworth, 1981). Remarkable reductions in palm damage and beetle population were observed within one to two years after the introduction of the virus (Bedford, 1986). In oil palm-producing countries, such as the Philippines, Indonesia, Maldives and India, the release of the *O. rhinoceros* virus has also been able to reduce the populations of the rhinoceros beetle (Zelazny and Alfiler, 1991; Zelazny *et al.*, 1992; Dhilepan, 1994). To fully exploit the potential benefit of the *O. rhinoceros* virus, the Malaysian Palm Oil Board (MPOB) initiated a project to develop a management programme for the rhinoceros beetle using the *O. rhinoceros* virus. The project was initiated in 1996, and was jointly conducted with AgResearch Limited, New Zealand, and later with several oil palm plantations in Malaysia. The significant findings of the project are reported and discussed in this article.

METHOD FOR VIRUS DIAGNOSIS

A simple and yet repeatable method, which is sensitive and specific for the diagnosis of the *O. rhinoceros* virus, was developed based on the polymerase chain reaction (PCR) technology (Richards *et al.*, 1999). Using a pair of DNA primers that specifically amplify the virus DNA, virus

infection is confirmed when a single DNA band of size 945 bp is visible on the agarose gel. As the virus DNA is extracted from gut tissues, PCR amplification can be inhibited when the DNA contains organic materials or soil. Therefore, optimisation is needed, before PCR can be routinely used for virus detection. A series of trials has successfully optimised the PCR method (Ramle *et al.*, 2001; 2010). The optimised concentrations of the PCR components are at 2.0 mM MgCl₂, 1.0 mM 10x PCR buffer, 0.2 mM each of Primers 15a and 15b, 0.5 U Taq-DNA polymerase and 0.4 mg bovine serum albumin (BSA). Addition of BSA (20 mg ml⁻¹) at 0.4 mg in the reaction increases the PCR sensitivity. The method is capable of detecting virus infection from DNA diluted 1 million times, or equivalent to a virus DNA concentration of as low as 2.23 pg µl⁻¹ (Figure 1).

A test to compare the sensitivity of the developed PCR method against the visual symptoms on infected beetles was conducted. Out of 839 gut samples with swelling and containing whitish milky fluid, 97.6% were diagnosed as infected. The PCR method was also capable of detecting the early stages of virus infection. From 307 adults that appeared healthy, 36.1% of them were found to be infected. The PCR method also detected 83.2% adult beetles from a pheromone trap to be infected by the virus; this was 13.6% higher ($P < 0.05$) than the detection rate based on gut morphological appearance (which was 69.6%) (Ramle *et al.*, 2010).

VIRUS INCIDENCE

A survey to determine the incidence of the *O. rhinoceros* virus in natural populations of the

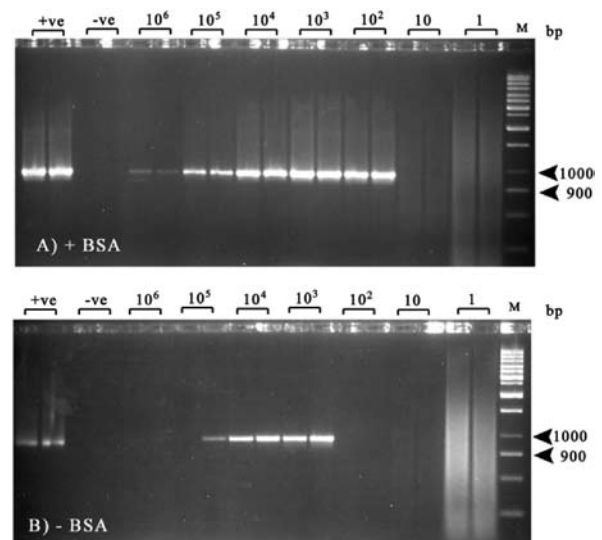


Figure 1. PCR amplification using a series of diluted *Oryctes* virus DNA. A) Reaction with addition of bovine serum albumin (BSA), and B) reaction without BSA. +ve = positive control; -ve = negative control; M = DNA marker.

O. rhinoceros beetle was conducted in 1999. Adults were sampled from pheromone traps and from breeding sites such as rotting trunks, fronds and empty fruit bunches of oil palm. The immature stages (first, second and third instars, pre-pupa and pupa) were sampled from the breeding sites. The samples were dissected and the gut tissues were collected for extraction of the *O. rhinoceros* virus DNA following the method by Ramle *et al.* (2010).

Virus Incidence in Adults

The results show that the adult beetles were commonly infected. The infection level, however, depended on the methods of collection and storage. Adults sampled from the pheromone traps and kept in a group had a higher virus incidence than adults that were kept individually separate. This suggests that transmission of the *O. rhinoceros* virus among the beetles occurred easily in the traps. Virus incidence in adults from the traps could reach as high as 98.1%, compared with adults that were kept individually (65%). Adults from the breeding sites had a much lower incidence level (21.8%) as they were possibly young adults, which were still free from virus infection (Table 1).

Virus Incidence in Immature Stages

In the larvae, the *O. rhinoceros* virus was diagnosed using gut tissues, as these tissues contained more virus particles than the haemolymph (gut tissue 20.9%, haemolymph 13.7%). Virus incidence increased as the larvae became older. Virus incidence in the first instar larvae (L1) was between 0% and 3%, in the second instar larvae (L2) from 0%-25% while in the third instar larvae (L3) it was from 30%-35% (Table 2). The life-span of each larva was a key factor that influenced virus incidence. A longer larval developmental period gave more time for the larvae to be exposed to the virus-contaminated materials in the breeding sites. The third instar larvae had the longest life-span (112.5 days), followed by the second instars (16.5 days) and first instars (15.5 days) (Bedford, 1980). The pre-pupae and pupae were free from the virus. Of the 20 pre-pupae and 17 pupae tested, none of

TABLE 2. INCIDENCE OF *Oryctes rhinoceros* VIRUS IN IMMATURE STAGES OF RHINOCEROS BEETLE

Developmental stage	Number of samples (N)	Percentage of virus infection (%)
L1	37	1.35 (0 – 2.7)
L2	81	12.5 (0 – 25)
L3	81	32.1 (29.2 – 35.0)
Pre-pupa	20	0
Pupa	17	0

Note: samples were collected from Sing Mah and Jendarata estates in Perak.

them were found to be infected (Table 2). The pre-pupa normally burrowed deep into the soil to form a cocoon, and this behaviour hindered contact with any form of material containing the virus. When the pupae successfully turned into new adults, they were mostly free from the virus.

GENETIC VARIATION IN THE VIRUS

The DNA of the *O. rhinoceros* virus extracted following the method of Ramle *et al.* (2005) was characterised by the endonuclease enzyme, *Hind*III. This enzyme has been used to characterise the *O. rhinoceros* virus from various regions in Asia (Crawford *et al.*, 1986). Four virus DNA profiles were identified and designated as *O. rhinoceros* virus types A, B, C and D (Figure 2). Type A was found to be identical to the virus DNA profiles of strain PV505, which were characterised by Crawford *et al.* (1985). Type A was commonly isolated from many localities in Malaysia (Figure 3). Insertion of a single band of size 15 471 bp differentiated type B from the other types (Wang *et al.*, 2008). The *O. rhinoceros* virus type B was previously isolated only from two sites, namely, Carey Island in Selangor and Bagan Datok in Perak (Ramle *et al.*, 2005), but now it has also been detected in Johor (Wang *et al.*, 2008). Type C has an insertion of a single band of size 2.0 kpb, and has only been isolated in Sabah. Type D has an insertion of two DNA bands of 15 471 kb and between 2.0 and 1.5 kpb, and was only isolated from a plantation in Kelantan (Figure 3).

TABLE 1. INCIDENCE OF *Oryctes rhinoceros* VIRUS IN ADULTS OF RHINOCEROS BEETLE USING DIFFERENT STORAGE METHODS

Sampling method	Method of storing samples	Number of samples (N)	Percent of virus infection (range)
Pheromone tapping	Kept in a group	206	74.6 (45.9 – 98.1)
	Kept individually	115	43.0 (31.4 – 65.6)
Breeding sites	Kept individually	56	21.8 (0 – 52.8)

Note: samples were collected from six plantations (Sedenak and Kempas Klebang in Johor, Sg Tekam in Pahang, Jendarata and Sri Pelangi in Perak, MAB in Selangor).

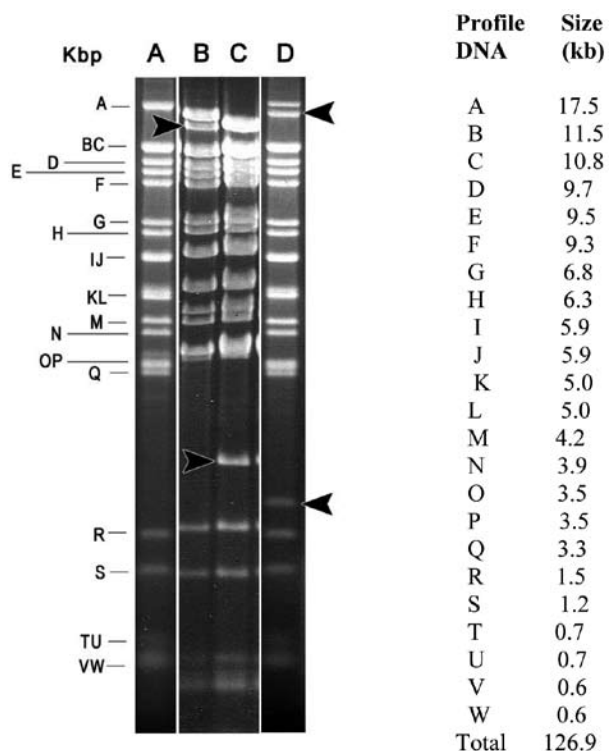


Figure 2. DNA profiles of *Oryctes rhinoceros* virus types A, B, C and D. DNA size was estimated based on a study by Crawford et al. (1985). Arrows show DNA fragments to differentiate virus types B, C and D from type A.

VIRUS VIRULENCE

The virulence of *O. rhinoceros* virus types A, B and C were bioassayed against third instar larvae (L3), newly emerged adults (neonates) and the field-collected mature adult beetles. The virus solution was freshly prepared from infected adults that

had gut tissues with advanced virus infection symptoms. Type A was prepared using samples from Sedenak Estate in Johor, the virus type B from West Estate in Selangor, while the virus type C was from Pitas Estate in Sabah. The gut tissues were macerated in sterilised millique water and centrifuged to sediment the tissue debris. The homogenate was filtered through a 0.45 µm cellulose membrane. The virus solution was stored at -20°C before the bioassays. The concentration of the virus solution was quantified by the PCR method, and standardised using a serial dilution method. Inoculation was done by placing onto the beetle's mouthparts 20-30 µl of the virus solution prepared in 10% sucrose. The bioassay results are shown in Table 3.

On the L3 larvae, the virus type B caused the highest mortality (86.9%), as compared with type A (26.7%) or type C (13.3%). The presence of the virus type B on the larval cadavers was also significantly higher than the presence of the virus types A and C. The LT₅₀ for the virus type B was 33.9 days, while for types A and C it was more than 100 days. In neonates, the virus type B again caused the highest mortality (86.7%) and infection (57.9%) as compared with the virus type A or type C. The LT₅₀ value of the virus type B was 36.8 days, shorter than those of the virus types A and C.

In mature adults, inoculation with the *O. rhinoceros* virus type A caused slightly higher mortality and infection than with the other types. The LT₅₀ value of type A was 33.1 days, shorter than for types B and C (Table 3). Mature adults responded differently to the *O. rhinoceros* virus as compared with larvae and neonates. Treatment with the virus type A caused a rapid increase in mortality.

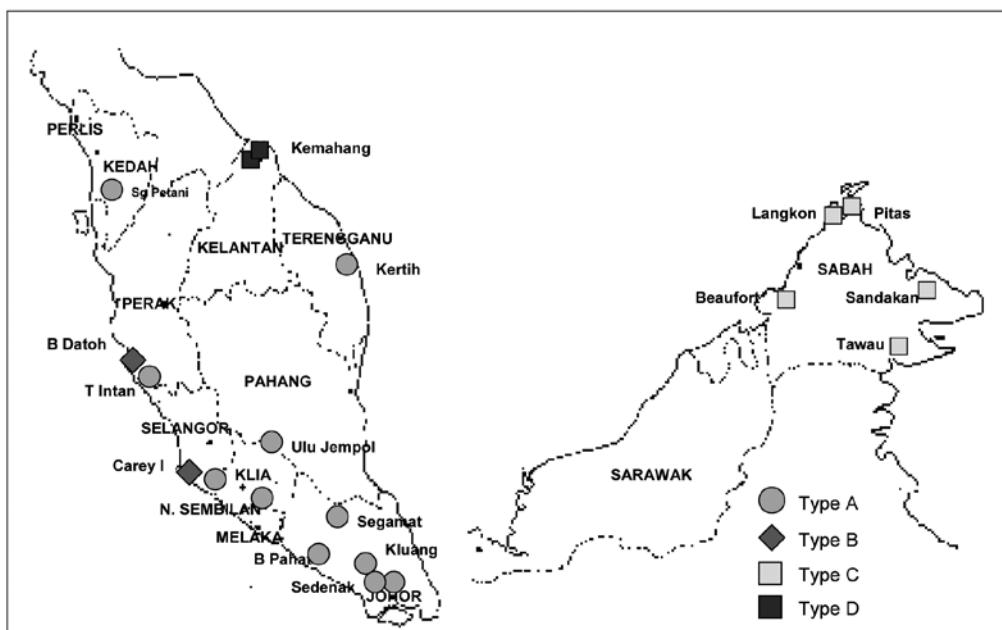


Figure 3. Geographical distribution of *Oryctes rhinoceros* virus types A, B, C and D in Malaysia.

TABLE 3. MORTALITY, INFECTION AND LT₅₀ VALUES OF *Oryctes rhinoceros* VIRUS IN LARVAE, NEONATES AND MATURE ADULTS OF RHINOCEROS BEETLE

Type of virus	Larvae *			Neonates *			Mature adults **		
	Mortality (%)	Infection (%)	LT ₅₀ (day)	Mortality (%)	Infection (%)	LT ₅₀ (day)	Mortality (%)	Infection (%)	LT ₅₀ (day)
A	26.7a	18.7b	> 100	63.7a	28.8a	48.58	50.0a	83.3a	33.12
B	86.7b	40.7a	33.95	83.3a	57.9b	36.89	45.2a	64.6a	62.38
C	13.3a	8.0b	> 100	10.7b	3.2c	> 100	29.9a	62.5a	86.35

Note: statistical analyses performed on data collected at *56 and **50 days after treatment. Mortality was calculated based on corrected mortality following Abbot's Formula. Means within a column with the same letter are not significantly different from one another at P>0.05 according to the Least Significant Difference Test (LSD).

At 30 days after treatment, mortality increased to 70.8%, *i.e.* significantly higher (P<0.05) than for the virus types B (10.4%) and C (9.4%). This high mortality, however, gradually reduced and was finally maintained at 50.0%, similarly with the virus types B and C. This finding shows that introduction of the same virus type as that already existing in the population can cause an additional impact on mortality. However, the impact seemed to be temporary, as mortality was eventually reduced and maintained at the same level as that of the other virus types. Marschall and Ioane (1982) who did repeated virus introductions to infected adult populations also reported almost similar findings.

VIRUS PRODUCTION

The commonly used method to mass-produce the *O. rhinoceros* virus was by an *in vivo* process, using the larvae of the rhinoceros beetle. The technique is simple and cheap, therefore, it has been used for the bulk production of the *O. rhinoceros* virus in the 1970s (Bedford, 1980). Larvae with advanced infection symptoms are easily recognisable. Their abdomens become translucent as the fat body disintegrates. The amount of haemolymph increases and this makes the larvae translucent when viewed against light (Figure 4a). For virus bulk production as carried out by Bedford (1980), healthy larvae (100-150 larvae) are reared in a box that contains a mixture of rotted sawdust and ground dead infected larvae. The infected larvae usually come to the surface of the substrate before death. In some cases the infected larvae have a prolapsed rectum (Figure 4b). These cadavers are collected daily and stored in a deep freezer.

In our research, an attempt to produce the *O. rhinoceros* virus types B and C *in vivo* was made following the method established by Bedford (1980). Healthy field-collected L3 larvae were inoculated and kept individually in cylindrical plastic cups till death. The presence of the virus was confirmed by PCR and the amount of haemolymph

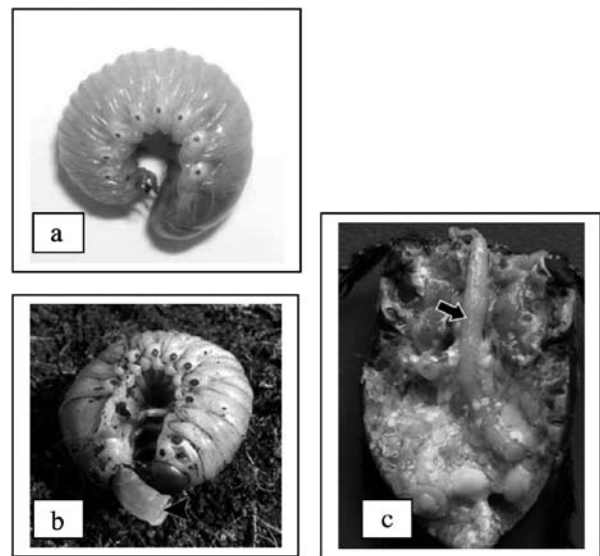


Figure 4. Infection symptoms of *Oryctes rhinoceros* virus in rhinoceros beetle. a) Infected larva became translucent when viewed against light. b) Infected larva with prolapsed rectum (arrowed). c) Infected adult with swollen gut tissues filled with milky content (arrowed).

was estimated. For the virus type B, out of 28 larvae, 17 larvae were confirmed to be infected, while for the virus type C, only four out of 25 larvae were confirmed to be infected. The average amount of haemolymph produced from each cadaver was 1.46 ml (N = 4), which was equivalent to 370 500 infective units (IU). At the recommended rate of inoculation of 1500 IU/adult, this amount can be used to inoculate a total of 245 adults.

The *O. rhinoceros* virus can be also produced from field-collected infected adults which have midguts showing advanced stages of virus infection (Figure 4c). The method to prepare the pure virus solution from either gut tissues or haemolymph is similar. Basically, the whole gut or haemolymph is transferred into a 1.5-ml tube, and then homogenised using a motorised micropestle until a cloudy solution is formed. The homogenate is centrifuged at 3000 rpm for 2-3 min to sediment the debris. The supernatant is filtered through

cellulosic membrane filters of 0.45 μm and 0.22 μm . The filtrate (solution with virus particles) is then stored at -30°C .

VIRUS INTRODUCTION SYSTEM

Introduction of the virus involves a series of activities. These include a pre-release site assessment, introduction of the virus, post-release monitoring and impact assessment. Details and objectives of each activity are elaborated as follows:

Pre-release Site Assessment

The objectives of this activity are to identify the existing type of the virus, to establish the infection levels in the adults and larvae before virus introduction, and to estimate the population density and the maturity of the adult population. Samples of adults (recommendation of more than 20 adults for each site) are collected from pheromone traps and placed individually in cylindrical plastic vials. The samples are dissected and the gut tissues collected. The morphological appearance of the guts is also recorded. The gut tissues are divided into two portions. The first one (a small portion, 1 cm long) is used for determining virus infection by PCR (Ramle *et al.*, 2010), while the remaining tissues are for virus characterisation by the restriction endonuclease enzyme *HindIII* (Ramle *et al.*, 2005). Virus infection in females, males and larvae is also determined. This information will be used as pre-release data.

In earlier virus introductions, the level of palm damage was monitored before and after the introductions (Purrini, 1989; Dhileepan, 1994). New and old damage to the palms due to rhinoceros beetle attacks are inspected. Damage in the first three fronds, the number of holes or cut leaves on old fronds and number of little leaves are estimated. The total number of fronds produced by each palm is also counted. Other activities such as the identification of the release site and the placement of pheromone traps in the release area are also conducted.

Introduction of the Virus

Introduction of the virus is by a capture-inoculate-release technique established by Zelazny (1978). Young healthy adults are collected using pheromone traps or from the breeding sites in the respective release sites. The young adults are commonly observed to be free from soil, having shiny elytra which are free from scratches. The collection should be conducted one to two days before virus introduction.



Figure 5. Inoculation of adults by placing virus solution on mouth-parts of adult beetles.

Prior to inoculation with the *O. rhinoceros* virus, the adults are placed in a container without food for 24 hr. This step will facilitate the inoculation process as the starved adults will readily and more speedily imbibe the virus solution. Virus inoculation is made on site by dropping the virus solution (20 μl prepared in 10% sucrose solution) onto the mouth parts of the beetles (Figure 5). Adults are released in the fields by placing them on the ground near the palm bases. Trapping activities and herbicide or pesticide applications are stopped for at least a month. This is to ensure that the inoculated adults are able to transmit the virus to other healthy adults in the population.

Post-monitoring and Impact Assessment

Adults are sampled from the designated pheromone traps while larvae are sampled from the breeding sites. All samples are placed individually in plastic vials so as to prevent cross-contamination during transportation and storage. For the first six months, the sampling activity is conducted monthly. Later on, it is conducted every three months until 24 months after the introduction. For every sampling, adults are collected over three or four consecutive trapping days. Samples are collected every morning and placed individually in plastic vials. Only 20% of the adults in the pheromone traps are sampled with the balance released back into the field. The capture is sorted according to the following parameters:

- population density of adults, proportion of males and sex ratio;
- virus infection in immature stages, males and females, as determined by the PCR technique; and
- the spread of the released virus, as determined by DNA analysis using the endonuclease enzyme *HindIII*.

Palm damage is estimated in the same locality as in the pre-assessment. The assessment is conducted every six months following the same method as in the pre-assessment.

INTRODUCTION OF VIRUS IN A YOUNG PALM AREA (less than one year after planting)

The *O. rhinoceros* virus type B was introduced in an estate with six-month-old palms. The area is peat, previously planted with cash crops such as pumpkin, yam, tapioca and maize. Beetle breeding materials on the ground were mostly rotten wood and decaying oil palm biomass, mainly empty fruit bunches. The level of virus infection in adults before introduction was 37.5%. The existing type of virus in the population was the *O. rhinoceros* virus type A. One hundred and fifty adults were inoculated and released in the centre of the release site. Adults were sampled using pheromone traps up to 15 months after release (MAR), while larvae were only sampled at 5 and 6 MAR. Assessment of palm damage was conducted twice, at 3 and 15 MAR.

Virus Infection and Population Density

The trends for virus infection and population density are shown in Figure 6. Virus infection increased gradually from 37.4% before the introduction and reached 100% between three and four MAR. Subsequently, virus infection declined slightly and was maintained at more than 90% until the experiment ended. Virus infection in the control plot, which was located 3 km away from the release site, also increased, but at a much slower rate. In the control plot, infection peaked at 100% at 11 MAR. For larvae at five MAR, out of 62 samples, only seven larvae were infected (11.3%), while at six MAR, of the six larvae samples, none were found to be infected. These findings support the results of previous work in many countries where the virus disease has already been established (Zelazny and Alfiler, 1991; Zelazny *et al.*, 1992; Dhileepan, 1994). It was found that *O. rhinoceros* virus infection was common among adult beetles, but occurred less frequently among larvae.

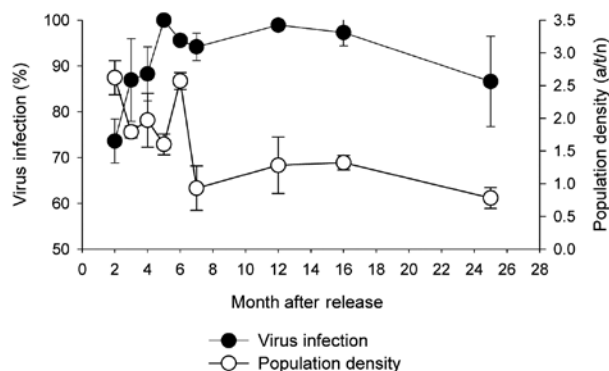


Figure 6. Percentage of *Oryctes rhinoceros* virus infection and population density of adult beetles in a young oil palm area.

The increase in virus infection and being maintained at a higher level affected the adult population (Figure 6). From the highest level of 2.62 adults/trap/night (a/t/n) at one MAR, the population was gradually reduced to 1.6 a/t/n at four MAR and to 0.93 a/t/n at six MAR, then slightly increasing again to 1.28 a/t/n at 11 and 15 MAR. There was a weak negative correlation between virus infection and population density ($Y=100.60 - 5.65x$, $R^2=39.74\%$, $F=2.72$, $P>0.05$), which was not statistically significant.

The number of males was also affected. The proportion of males was reduced from 43.5% at two MAR to the lowest level of 28.7% at 4 MAR, and then maintained at about 30%-37%. A similar declining trend in males had also been recorded in the Maldives: the number of males reduced from 63.2% to the lowest level of 50.9% at six to 12 MAR, and was then maintained at 58.4% at 24 MAR (Zelazny *et al.*, 1992).

Virus Establishment and Palm Damage

Establishment of the released *O. rhinoceros* virus type B in the experimental site was observed as early as at three MAR (Figure 7). The DNA profiles of the virus type B were detected in the inner region of the site at three MAR, and then spread to the middle and outer regions, finally with the virus established in the control plot at 11 MAR. The virus persisted at high levels as type B DNA profiles were observed again at 15 MAR. This supports the results that the rapid increase in virus infection at three to four MAR was due to infection by the released virus type B. Average palm damage was significantly reduced ($P<0.05$) from 11.2% to 4.0% at 15 MAR, and then decreased further to 3.4% at 24 MAR (Table 4). This level of control is considered satisfactory, as there was no application of any chemical insecticides throughout the whole period of study.

INTRODUCTION OF VIRUS IN AN IMMATURE PALM AREA (more than three years after planting)

The *O. rhinoceros* virus type B was introduced in an estate with three-year-old palms located in Johor. The estate practiced the zero burning replanting technique. A total of 125 infected adults (90 females and 35 males) were released. The prevailing virus was type A. The experimental site was divided into three release regions, namely, inner (IR), middle (MR) and outer regions (OR). Pheromone traps were placed at the boundaries of each region. At the time of release, most of the rotting oil palm residues on the ground were fully covered by the leguminous cover crops.

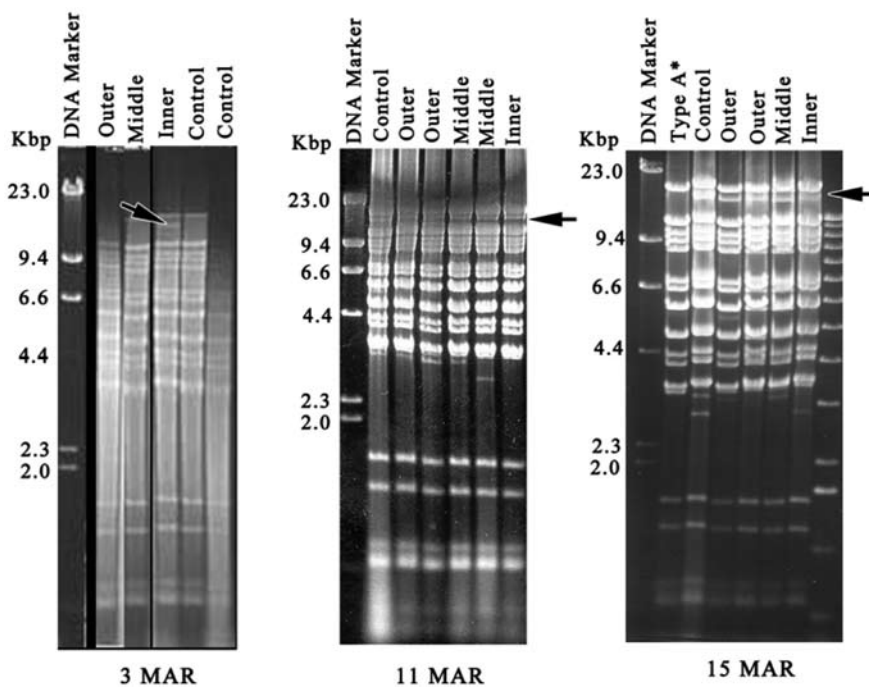


Figure 7. Establishment of released virus type B (arrowed) in a young oil palm area up to 15 months after release (MAR).

TABLE 4. PALM DAMAGE IN A YOUNG PALM AREA AFTER INTRODUCTION OF *Oryctes rhinoceros* VIRUS

Month after release	Palm damage (%) in each release region (Mean ± SE)			Mean ± SE
	Inner	Middle	Outer	
3	16.2 ± 3.7a	9.4 ± 2.3a	8.0 ± 3.0a	11.2 ± 3.7b
15	5.1 ± 1.0b	4.3 ± 1.0a	2.6 ± 0.5a	4.0 ± 2.3a
24	2.8 ± 0.9b	4.2 ± 0.9a	3.1 ± 1.3a	3.4 ± 3.0a

Note: means within a column with the same letter are not significantly different from one another at P>0.05 according to the Least Significant Difference Test (LSD).

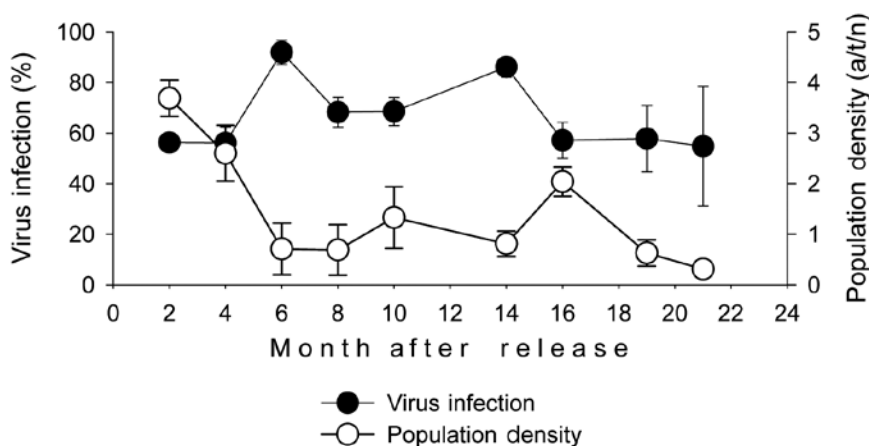


Figure 8. Impact of released *O. rhinoceros* virus type B on infection level and population density of rhinoceros beetles in an immature area in Johor.

Virus Infection and Population Density

Virus infection increased slowly and peaked at 100% at six MAR, then reduced gradually and was maintained at 60%-70% for a period of eight months, before declining to below 40% (Figure

8). Such an increase in virus infection right after introduction was commonly reported in many previous studies in the Pacific islands (Zelazny, 1973, 1977; Marschall and Ioane, 1982). Other virus release studies conducted in the Philippines (Zelazny and Alfiler, 1991) and Maldives (Zelazny

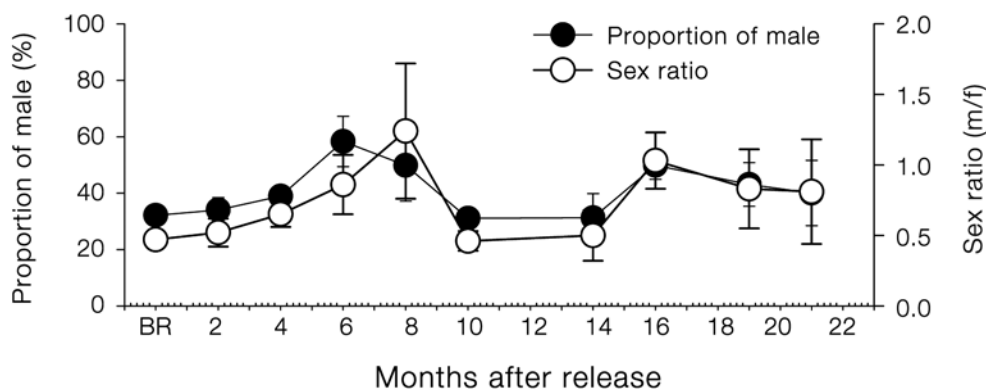


Figure 9. Proportion of males and sex ratio of rhinoceros beetles sampled from an immature area in Johor. BR = before release.

et al., 1992) showed that the highest level of infection at 30%-32% was recorded as early as three to nine MAR, then gradually reduced to about 10%-15% at 20 MAR.

Trends of repeated fluctuations in the adult beetle population were commonly observed in oil palm plantations (Ho, 1996). The adult beetle population peaked six times within 12 months of a study period (Norman and Mohd Basri, 2004). In this current study, the adult beetle population only peaked twice, most likely because of the introduction of the virus. The population reduced from the first highest peak at two MAR to the lowest level at four to six MAR, and was then maintained at low levels for a certain period of time before slowly increasing again to reach the second peak at 16-18 MAR. In this case, the population was maintained at a low level (0.71-1.33 a/t/n) for a period 10 months (Figure 8). This result supports the findings by Marschall and Ioane (1982) who released the *O. rhinoceros* virus in Western Samoa. The results show that the beetle population was reduced as early as at two to three MAR, and then gradually increased to a higher level at four to five MAR. Although there was a trend of negative correlation between virus infection and population density, the association was weak. This suggests that the reduction in beetle population at the release sites was also influenced by other factors.

Proportion of Males and Sex Ratio

The proportion of males is a key parameter used by other researchers to estimate the impact of a virus release programme. More males are normally infected than females (Zelazny *et al.*, 1992). The natural behaviour of the males in staying longer at the breeding sites preparing vicinity for females to lay eggs, exposed them more to the contaminated substrates. Females spend more time searching the oil palms for food before mating and laying eggs. In this study, the reduction of males occurred only in later stages, *i.e.* at three to four months after

the virus introduction (Figure 9). This delay was possibly because the infected adults required time to transmit the virus to other healthy ones. In the field, the infected adults need at least two months to transmit the virus to other healthy partners (Gorick, 1980). The slower trend in reduction may possibly be related to a slower virus transmission process among the adults, as they may have already adapted to the virus. This phenomenon has been recorded in countries where the virus exists naturally, such as in the Philippines (Zelazny *et al.*, 1989). Sex ratio has a significant positive correlation with the number of males, and, therefore, both parameters can be used to monitor the impact of a virus introduction programme.

Virus Infection in Males and Females

The *O. rhinoceros* virus mainly affects the adult stages rather than the larval stages. An infected adult has a shorter life-span, stops feeding and dies four to five weeks earlier than its natural maturity period (Zelazny and Alfiler, 1991). In this study, virus infection in males and females was not significantly different (Figure 10). The infection level of males, however, was slightly higher than the females for a period up to 16 MAR. These infected males were possibly older ones, as male adults had been reported to be more frequently infected than females (Zelazny, 1977; Zelazny *et al.*, 1992). The females which were mostly newly emerged were still free from virus infection.

Females are more susceptible to the *O. rhinoceros* virus than males (Zelazny, 1973). Infected females die earlier and produce fewer eggs. A reduction in the number of females increases the proportion of males and also the sex ratio. This is possibly one factor that may contribute to a slower reduction in both parameters as well as in the population density in the early stage of three to six MAR. Meanwhile, the reason why females are more susceptible than males is still unknown and needs further investigation.

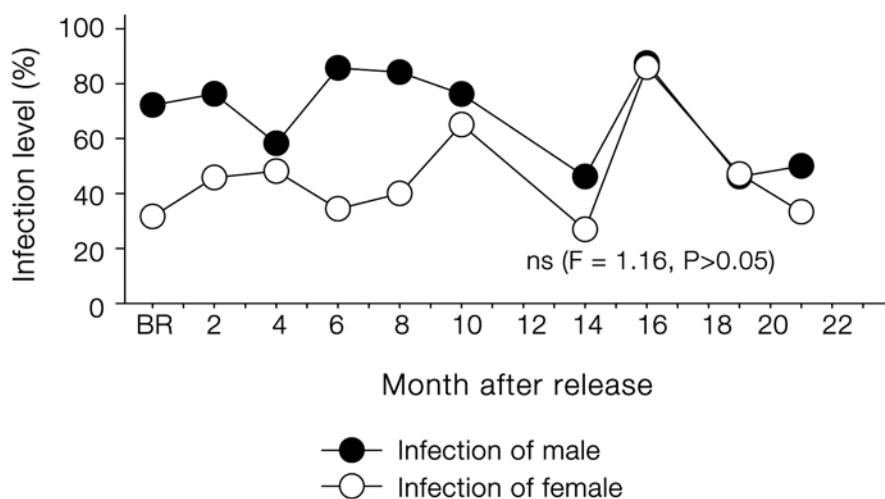


Figure 10. Infection by *Oryctes rhinoceros* virus of male and female rhinoceros beetles sampled from an immature area in Johor. BR = before release.

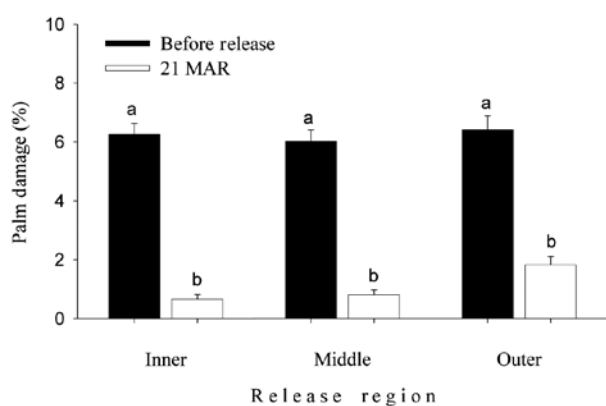


Figure 11. Percentage of palm damage in an immature area in Johor. Bars with the same letter are not significantly different from one another at $P > 0.05$ according to the Least Significant Difference (LSD) test, MAR = months after release.

Virus Establishment and Palm Damage

Introduction of the *O. rhinoceros* virus type B into a population of adults already infected with type A virus was successful in further reducing the adult population and as well as palm damage. Palm damage was reduced to below the threshold level of 5% (Figure 11). Reductions in palm damage have also been reported in previous virus introduction studies, such as those carried out in Western Samoa, Willis Islands and India (Bedford, 1980; Dhileepan, 1994).

The DNA profiles of the released type B virus were recorded in the inner release region at four MAR (Figure 12). The short establishment time of the released *O. rhinoceros* virus type B in this estate was possibly related to the high population of adults (3.69 a/t/n) and the lower infection level of females (31.6%). A study by Ramle *et al.* (2005) who introduced the *O. rhinoceros* virus type B virus in an area with a high population density showed that

the virus was spread as early as three MAR, and was later established in the whole experimental area by 11 MAR.

FACTORS INFLUENCING THE SUCCESS OF VIRUS TRANSMISSION

Previous work showed that introduction of the *O. rhinoceros* virus in the virus-free areas of coconut-producing countries successfully controlled the rhinoceros beetle and reduced palm damage. The released virus established in the beetle population and spread to other parts within a year after introduction (Marschall, 1970; Bedford, 1986). Introduction of the virus in areas where the virus was already established, such as in India, Maldives, Indonesia and the Philippines, was able to further reduce the rhinoceros beetle population as well as palm damage (Marschall and Iaone, 1982; Zelazny *et al.*, 1992; Dhileepan, 1994). The disease drastically reduced the life-span of the adults; however, there were less effects on the overall survival of the larvae (Zelazny *et al.*, 1992).

Based on previous and current studies, it may be inferred that successful introduction of the *O. rhinoceros* virus in the field is influenced by various factors. Among them, three factors are identified which could possibly play a key role in ensuring the successful transmission of the released virus in the population. These factors are the adult population density, an abundance of breeding sites and the maturity of the adult population. Successful transmission of the released virus in the population depends on adult density, while adult density is influenced by an abundance of breeding habitats (Zelazny and Alfiler, 1991). As transmission of the *O. rhinoceros* virus is mainly via mating, the higher the adult population, the higher

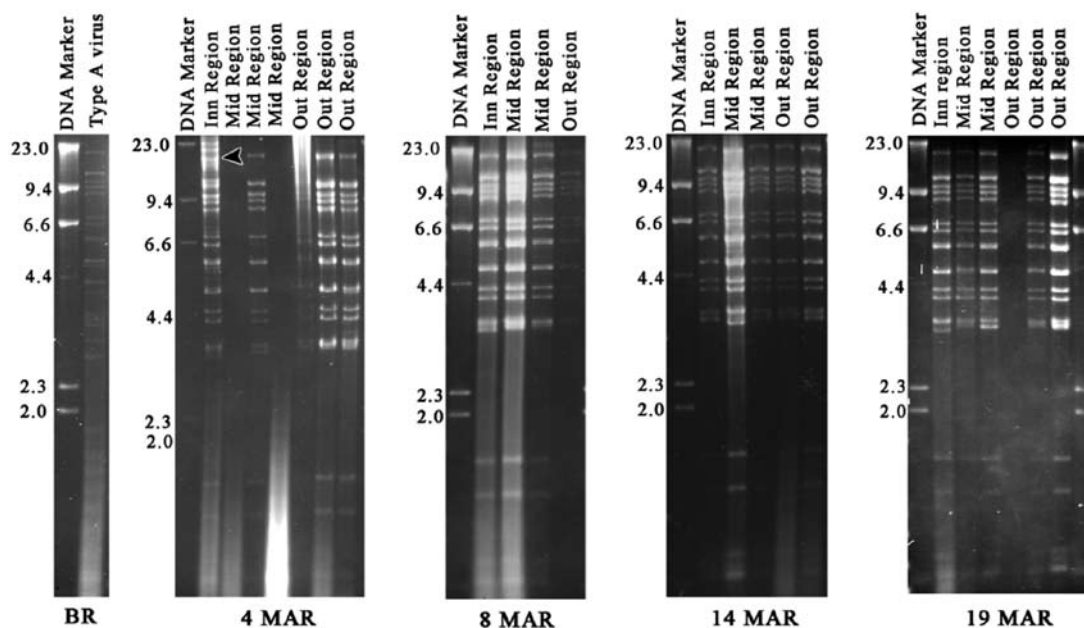


Figure 12. DNA profiles of *Oryctes rhinoceros* virus sampled from an immature area in Johor before release (BR), and at 4, 8, 14 and 19 months after release (MAR). Virus DNA was digested by an endonuclease enzyme *Hind*III. A single band (arrowed) of 15 471 bp was used to confirm the presence of the released virus type B at four MAR in the inner region of the release site.

the chances of mating (Zelazny, 1976). As mating commonly occurs in the breeding habitats (Zelazny and Alfiler, 1991), the availability of breeding sites is, therefore, one of the key elements to ensure the successful spread and establishment of the released virus in the field.

Another key factor is the density of young adults, which are commonly still virus-free and are easily infected by the virus (Zelazny and Alfiler, 1991). Our bioassay results show that the neonates are more susceptible than the older infected adults. Introduction of the virus in an area with a high young adult population results in the successful spreading of the released virus and then in reducing the adult population as well as palm damage (Ramle *et al.*, 2005). High numbers of infected adults, especially the older ones, may limit virus transmission and subsequently restrict the establishment of the disease epizootic in the population. The older adults have possibly already adapted to the virus infection and may possess some degree of tolerance. This study shows that even when the percentage of infected adults was maintained as high as 70%-100% for several months, it caused only a small effect on the population. This phenomenon may have occurred in Malaysia because the virus has existed naturally in the beetle populations for more than 40 years (Huger, 1966). The incidence of beetle adults tolerant to virus infection has been also reported in the Philippines (Zelazny *et al.*, 1989).

The use of a method of diagnosis to determine the presence of the released virus in the field is possibly another factor that can contribute

to include the hitherto undetected presence of the released virus. For example, in this current study, restriction of virus DNA by the endonuclease enzyme *Hind*III required a higher yield of DNA in order to successfully diagnose the presence of the released virus. Therefore, the virus spread may not be detected if the amount of virus DNA was too low. A more sensitive method of diagnosis is therefore required especially to monitor the spread of the released virus in the field. The PCR-based technology using specific DNA markers is proposed as it is simple and requires only low amounts of DNA (Ramle *et al.*, 2010).

FUTURE RESEARCH

Screening of effective types of the virus should be continued to cover the whole collection of samples, not only from oil palm but also from other crop areas. Attempts at collecting and screening the *O. rhinoceros* virus from various research institutions such as those in India, the Philippines, Indonesia, the South Pacific islands and Africa can probably detect more highly virulent virus types. A standard bioassay system should be established among laboratories for estimating the virulence of the samples in the *O. rhinoceros* collection.

The use of insect cell culture, such as cell line DSIR-HA-1176, has proved to be suitable for the replication of the *O. rhinoceros* virus (Crawford, 1982; Crawford and Sheehan, 1985). Cell line DSIR-HA-1179 has also facilitated genetic studies such as construction of the virus physical map of the

O. rhinoceros virus from 12 different geographical origins (Crawford *et al.*, 1985; 1986). This virus genetic variation can help in the taxonomy and identification of the effective types of the *O. rhinoceros* virus (Jackson *et al.*, 2005). Besides being used to produce higher yield and a better quality of the *O. rhinoceros* virus, this insect cell culture also can be employed to estimate the effect of multi-infections among the virus types.

Specific DNA primers for each Malaysian and other types of the *O. rhinoceros* virus are required and need to be developed. Using the PCR technique, the specific primers can be used in post-monitoring to track virus transmission and simultaneously differentiate between the released virus from the existing virus in the population. Furthermore, this rapid and accurate method only requires a low yield of DNA in order to detect the presence of the virus (Ramle *et al.*, 2010). This new method of diagnosis can perhaps determine the role of the released *O. rhinoceros* virus, especially when it is incorporated into a control programme together with other pathogens.

Generally, in areas where the presence of the *O. rhinoceros* virus does not affect the adult population, it is recommended to use the fungal pathogen *Metarhizium anisopliae* to kill the larvae population (Ramle *et al.*, 2007). Questions of whether there would be a conflict between the *O. rhinoceros* virus and *M. anisopliae*, or if there was any possibility of virus transmission breakdown resulting in the loss of the virus from the system, can be answered using the PCR diagnosis method. More importantly, the findings from this study will also be used to develop an effective integrated bio-management system of oil palm against the rhinoceros beetle.

CONCLUSION

The development of a PCR-based diagnosis method makes the detection of the *O. rhinoceros* virus in the rhinoceros beetle simpler and more accurate. Adult beetles are more commonly infected than the larvae, while pre-pupae and pupae are free from the virus. Four types of the *O. rhinoceros* virus had been identified and were referred to as *O. rhinoceros* virus types A, B, C and D. Type B was more pathogenic than the other types, therefore, it was chosen for the field release studies. The virus was produced *in vivo*, using the third instar larvae or adult beetles. The virus inoculum was prepared by centrifugation and filtration methods, and then formulated in 10% sucrose. A field introduction system of the virus was established, which involved a pre-release site assessment, introduction of the virus in the field, and post-monitoring and impact assessment. The first virus release study was carried out by introducing

the *O. rhinoceros* virus type B in a young oil palm area with existing virus type A. The released virus successfully reduced the adult population as well as palm damage. Virus establishment was detected as early as three MAR and persisted up to 15 MAR. A high density of young adults, a low infection level in old adults and an abundance of breeding habitats may have been the factors which ensured the successful transmission of the released virus in the study area.

Results of the virus release study in an immature palm area show that the increase in virus infection affected the adult population. However, the population was reduced at a slower rate before infection was maintained a low level for a certain period of time. A weak negative correlation between virus infection and adult population suggests that the virus played only a small role in influencing the beetle population. The proportion of males was reduced at a slower rate, which was possibly due to slow virus transmission because they may already have adapted to the virus. Analysis of DNA showed that the *O. rhinoceros* virus type B virus was only established at four MAR, and was not detected until the experiment ended at 22 MAR. Findings from these field studies suggest that introduction of the *O. rhinoceros* virus in the early stages of replanting was more effective in controlling the rhinoceros beetle. To fully utilise the virus for better control of the rhinoceros beetle, further research is needed, especially to get higher virulence among the virus types, to produce better virus quality, to establish a stable formulation, and to develop the PCR-based technology for more reliable and for specific virus monitoring. Integration methods between the introduction of the *O. rhinoceros* virus and the fungus *M. anisopliae* also need further investigation.

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