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SHORT COMMUNICATION

An Opportunistic Bacterial Pathogen, *Pseudomonas alcaligenes*, May Limit the Perpetuation of *Oryctes* Virus, a Biocontrol Agent of *Oryctes rhinoceros* L.

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Pseudomonas alcaligenes *was detected at a high concentration (10^9–10^{10} cells mL^{-1}) in the haemolymph of some dead* Oryctes rhinoceros *grubs collected from its breeding sites in the three southern districts, viz. Alleppey, Quilon and Kottayam of Kerala State, India. In a laboratory colony maintained for production of* Oryctes *virus, an important biocontrol agent of this major coconut pest, approximately 52% of the grubs succumbed to septicaemia with similar symptoms. The bacterium was found to be a component of the gut microflora of healthy grubs. Occurrence of the viral infection naturally or when induced in the laboratory in the* O. rhinoceros *grubs, appeared to be one of the biotic stress factors for* P. alcaligenes *to become an opportunistic pathogen. A preponderance of this bacterial infection in field populations during the periods when natural viral infection in grubs was above average, agrees with this observation. This finding becomes significant as infection by the opportunistic bacterial pathogen,* P. alcaligenes, *reduces the production of* Oryctes *virus inoculum in nature and limits the field-perpetuation of this viral biocontrol agent.*

Keywords: *coconut, opportunistic pathogen,* Oryctes rhinoceros, Oryctes *virus,* Pseudomonas alcaligenes

One of the major ubiquitous pests infesting coconut gardens is the rhinoceros beetle, *Oryctes rhinoceros* L. The direct and indirect damage caused by the adult results in approximately 10% economic loss to the growers (Nair, 1986). Additionally, the wounds made by the adult beetles serve as portals for the entry of the red weevil, another pest of coconut, and important disease causing fungi. The immature stages of this pest are found in farmyard manure, compost, coir waste, decayed fallen coconut trunks and standing coconut stumps. A comprehensive biological control of this insect pest has been reported by using a non-occluded virus, *Baculovirus oryctes* (Bedford, 1976; Mohan & Pillai, 1993), which has now

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been transferred to its own family and is called *Oryctes* virus based on its ds DNA genome and specificity to single family of Coleoptera (Evans & Shapiro, 1997).

The maintenance and multiplication of this virus is possible *in vivo* in *O. rhinoceros* grubs and adults, for which a steady stock of the healthy hosts is necessary. Therefore, field collections were carried out frequently from three southern districts of Kerala, India. Such collections always yielded some dead *O. rhinoceros* grubs. These grubs did not show any gross symptom of natural virus infection (Zelazny, 1972; Mohan *et al.*, 1983). In the laboratory also, certain healthy grubs which were inoculated with virus for culture maintenance and multiplication resulted in a similar type of mortality as observed in the field-collected samples. Isolation made from both field-dead as well as *Oryctes* virus-inoculated lab-reared dead grubs resulted in the growth of a single type of bacterial colony. This paper discusses the significance of the presence of this bacterium and its ramifications in the control of rhinoceros beetle using *Oryctes* virus.

For preparing *Oryctes* virus inoculum, virus-diseased *O. rhinoceros* grubs were dissected and the midgut epithelium was removed and placed in 1% (w/v) sodium thioglycollate at 4°C, to prevent discolouration by melanization. All the steps were carried out at 4°C over ice. The suspension was then triturated and filtered through muslin cloth. The filtrate was centrifuged at $2000 \times g$ for 10 min and the resulting supernatant at $50\,000 \times g$ for 1 h. The pellet was resuspended in 1% thioglycollate and clarified (Payne, 1974). The clarified suspension was then aseptically passed through 0.45 μm millipore membrane filter to eliminate bacteria. Further, the filtrate was tested by streaking on nutrient agar plates to ensure that the suspension was free of any bacterial contamination. The viral inoculum thus prepared was used for the studies.

Field collections of the *O. rhinoceros* grubs and adults were done fortnightly from coconut gardens in Krishnapuram, Kappil, Purakkad and Thotappally areas in Alleppey district and Ayiramthengu, Oachira, Choonad, Thodiyoor, Thazhava and Karunagappally areas in Quilon district and Vazhappally area in Kottayam district of Kerala State, India from September 1996 to August 1999. From these collections, healthy grubs/adults were segregated and maintained in the laboratory in sterilized, moist (40–50% moisture) coir waste (Gopal & Sathiamma, 2000). Maintenance of the virus culture was done by force-feeding 250 μL of viral inoculum to the healthy grubs. Both healthy and virus inoculated insects were observed daily for any sign of abnormal changes.

All the dead grubs were screened for virus disease or other infections. Insects which died with symptoms other than virus infection were surface sterilized using 0.1% $HgCl_2$ (2–4 min) and 90% (v/v) ethanol (5 min) and then thoroughly washed with sterile distilled water. They were then dissected and the haemolymph was aseptically drawn into a sterile syringe. The syringate was serially diluted in sterile water and plated on nutrient agar (NA) as well as potato dextrose agar (PDA) medium and incubated at 28°C. Midgut and fat bodies were also plated. Resultant colonies were purified and maintained on NA/PDA slants. Similarly, the haemolymph of field-dead and healthy grubs was also serially diluted and plated. Simultaneously, the presence of virus infection was also checked by staining smears of midgut epithelium and haemolymph using 3% Giemsa stain (Mohan *et al.*, 1983).

Identification of the bacterial isolates was done as per Holt *et al.*, 1994. The isolates were tested for the following characteristics: colony and cell morphology; Gram reaction; motility; oxygen dependence; spore formation; cytochrome oxidase and catalase activity. The isolates were further tested as follows: formation of fluorescent/non-fluorescent, diffusible/non-diffusible pigments, breakdown of starch, gelatin liquefaction, H_2S production, denitrification, production of arginine dihydrolase, urease activity, decarboxylation of lysine, formation of indole from tryptophan, growth at 4 and 41°C. These tests were performed as described by Conn and Pelczar (1957), Collins and Lyne (1976), and Cappuccino and Sherman (1992).

For the pathogenicity bioassay, apparently healthy field-collected grubs of *O. rhinoceros* were maintained in sterilized coir waste in laboratory for up to 20 days and from these, the highly active third instar grubs with no external evidence of any disease, were selected. Four

treatments were included in the bioassay. In the first treatment, *O. rhinoceros* grubs were force-fed with 0.25 mL of *P. alcaligenes* inoculum grub (bacterial inoculum concentration 1×10^8 cells mL^{-1}). The second treatment included force-feeding the grubs with 0.25 mL of *Oryctes* virus inoculum, which was prepared as described earlier. In the third treatment, the grubs were force-fed with 0.25 mL of *Oryctes* virus inoculum and 24 h later with 0.25 mL of *P. alcaligenes* inoculum (1×10^8 cell mL^{-1}). The fourth treatment was maintained as control, in which the grubs were force-fed with 0.25 mL of sterile distilled water. All the treatments were replicated five times with 10 third instar grubs in each replication, which were monitored at 24 h intervals. Symptoms of mortality were recorded daily. Haemolymph from all the dead grubs (either due to bacterial or virus infection) was plated to confirm the presence of the same bacterium. Giemsa staining of the midgut epithelium was also simultaneously carried out for detecting the presence of *Oryctes* virus.

The collection of *O. rhinoceros* grubs (6627 in number) and adults (307 in number) from farmers' coconut gardens, during September 1996 to August 1999, revealed about 5–10% dead grubs which were dirty brown in colour and presumably would have died of bacterial infection. Of these field collected grubs, 20% (SEM 4.22) succumbed to bacterial infection when maintained in the laboratory in sterilized coir waste. In contrast to this, 52% (SEM 4.58) of grubs inoculated with virus and reared in the laboratory died of bacterial infection when maintained in sterilized coir waste. Under the light microscope, smears of tissue scraped from the midgut epithelium of such dead grubs, when stained with 3% Giemsa, showed sparse presence of hypertrophied nuclei which, however, could not be detected in the haemolymph smears.

The onset of bacterial infection was seen in the laboratory with the live grubs turning shiny and transparent with the darkly coloured midgut clearly visible. The bodies of the grubs became soft yet retained their shape. Within 12 h, the grubs became light yellowish in colour. In the next 12 h, the whole body turned dirty brown as the grubs reached the moribund stage. Chalky white spots were observed in the last few segments of the abdomen. A very distinct feature of this infection was the presence of an unpassed dry lump of faecal pellet in the partially opened rectum. When picked up with a pair of forceps, the flaccid dead body of the grub hung like a loose skin bag filled with fluid.

On dissection, a foul odour emanated from such grubs. These infected grubs had an increased haemolymph content, which was slightly brown in colour. Fat bodies were putrefied into chalky white spots. The midgut looked dry with hardened food material inside. Plating the haemolymph, midgut and fat bodies on NA or PDA medium gave rise to singular type of bacterial colonies within 24 h of incubation. The discrete colonies were round, shiny with an entire margin, and slightly yellowish in colour. The culture plates released a distinctive odour when slightly opened. Similar colonies were found to grow amongst other types of bacterial colonies, when midgut content from healthy grubs was plated. In both healthy and virus-infected adult beetles, the abdominal contents contained predominantly yeast-like microorganisms.

The bacteria isolated from field-collected dead grubs (BI 1), healthy laboratory-reared dead grubs (BI 2), virus-inoculated laboratory-reared dead grubs (BI 3) and healthy live grubs (BI 4) were Gram-negative, short rods, non-sporulating, motile, aerobic, catalase and oxidase positive, lacking diffusible or nondiffusible, fluorescent or non-fluorescent pigments, gelatinase, urease and arginine dihydrolase positive and grew at 41°C (Table 1). These characteristics indicate that these isolates belonged to *Pseudomonas alcaligenes*. The bacterium, *P. alcaligenes*, was also isolated from healthy grubs in low numbers. The bacterial count in the midgut of healthy grubs was 10^2–10^3, which significantly increased in moribund stages. The infected grubs harboured around 10^9–10^{10} cells mL^{-1} in the haemolymph.

Transmission of *Oryctes* virus in nature in *O. rhinoceros* takes place from infected beetles to healthy beetles and immature stages, and from diseased immature stage to the healthy beetles (Zelazny, 1976). Previously, 54% of beetle populations in southern parts of Kerala, India were infected (Mohan *et al.*, 1983). Recent 3 year's field collection data revealed that

TABLE 1. Morphological, physiological and biochemical tests of bacterial isolates BI 1, BI 2, BI 3 and BI 4

Test	Observation
Morphological tests	
Colony morphology	Round, shiny, entire margins, slightly yellowish in colour
Pigments	None
Gram's reaction	Negative
Cell Shape	Short rods
Spore formation	No
Capsule	No
Motility	Positive
Flourescence (UV)	Negative
Physiological	
Growth at temperatures	From ambient to 41°C; no growth at 4°C
Growth under anaerobic condition	Negative
Biochemical tests	
Cytochrome oxidase	Positive
Catalase activity	Positive
Gelatin liquefaction	Positive
Oxidation/fermentation (O/F)	Negative
H_2S production	Negative
Urea hydrolysis	Positive
Amylase production	Negative
Lysine decarboxylase	Positive
Arginine dihydrolase	Positive
Indole production	Negative
Utilization of L-arginine	Positive

5% (SEM 2.65) of the 6627 grubs and 22% (SEM 1.55) of 307 adults collected from the field were infected by *Oryctes* virus, indicating a 32% decrease in adult infection. One of the reasons could be the failure of healthy beetles visiting virus-infected sites or the failure of diseased beetles to deposit excreta in places where healthy beetles and grubs are present. The presence of a high count of an opportunistic bacterial pathogen, *Pseudomonas alcaligenes*, in field-collected dead grubs and virus-inoculated laboratory-reared dead grubs indicate another potential cause of loss of natural viral inoculum. Midgut epithelium smears of the field-collected dead grubs consistently revealed the presence of hypertrophied nuclei, an important histopathological symptom of virus infection, indicating that there was a certain level of viral disease development in the grubs before they succumbed to septicaemia.

The pathogenicity bioassay results (Table 2) showed that when the isolated bacterium was force-fed to grubs inoculated with virus, high mortality occurred due to bacterial infection within 8–12 days. Some of the grubs which received viral inoculum alone also succumbed to bacterial infection in 10–15 days time, implying that the resident bacterium in the gut gets a favourable micro-environment to multiply and cause infection, though in this case, the time taken was slightly longer. *O. rhinoceros* grubs which died of typical *Oryctes* virus disease showed very high numbers of hypertrophied nuclei in the midgut epithelium, when stained with 3% Giemsa solution. In the grubs inoculated with *Oryctes* virus, but dying of *P. alcaligenes* caused septicaemia, hypertrophied nuclei could be detected in the midgut epithelial smears but in very low numbers, resembling the cytological results of field-collected dead grubs. Bacterial inoculation alone could not elicit high mortality in the insects demonstrating the limited ability of *P. alcaligenes* to kill on its own. Haemolymph from septic grubs, when plated, produced identical bacterial colonies. Grubs which survived virus/bacterial infection, when plated, produced a multitude of bacterial colonies with very few resembling *P. alcaligenes*. This laboratory experiment lends credence to our observation that

TABLE 2. Effect of *Pseudomonas alcaligenes* (PA)/*Oryctes* virus (OrV) inoculation in grubs[a] of *Oryctes rhinoceros*[b]

Treatments	% Total mortality	% Mortality due to PA	Presence of PA in H	Presence of PA in M	Presence of Hypertrophied nuclei in H	Presence of Hypertrophied nuclei in ME	% Mortality due to OrV	Presence of PA in H	Presence of PA in M	Presence of Hypertrophied nuclei in H	Presence of Hypertrophied nuclei in ME
Control	08	00	−	+	−	−	08	−	−	+	+
PA	20	20	+	+	−	−	00	−	+	−	−
PA + OrV	96	60	+	+	−	+	36	−	−	+	+
OrV	92	48	+	+	−	+	44	−	−	+	+
Gen. Mean	54	32					22				
SEm	10.21	11.40					7.91				
CD ($P=0.05$)	14.06	15.71					10.89				

[a]Third instar larval stage.
[b]Values are means of five replications of 10 grubs each.
H = haemolymph; M = midgut; ME = midgut epithelium.

P. alcaligenes acts as an impediment in the perpetuation of *Oryctes* virus in the natural population of *O. rhinoceros*.

Pathogenesis of *O. rhinoceros* grubs by the opportunistic bacterium *P. alcaligenes* is the first report of its kind. *Acinetobacter calcoaceticus* has been previously reported to kill *Oryctes* (Kannan *et al.*, 1980), although trials with bacterial pathogens have been unsuccessful (Surany, 1960; Zelazny, 1971). However, there are reports of bacterial infections in other scarab beetles such as turf and pasture pests. A spore-forming bacterium, *Paenibacillus popilliae* (previously *Bacillus popilliae*), an obligate parasite, invades the haemolymph of scarab larvae, such as the Japanese beetle *Popilliae japonica*, and produces milky disease (Klein & Jackson, 1992). Similarly, *Serratia entomophila* and *S. proteamaculans*, non-spore forming bacteria, cause amber disease in the grass grub *Costelytra zealandica* (Jackson & O'Callaghan, 2000) and have been marketed (INVADE™, Monsanto) in New Zealand for the control of this serious turf pest (Jackson *et al.*, 1992).

The death of grubs from this secondary bacterial infection, particularly in those where virus is multiplying, is disadvantageous since it depletes the production of natural viral inoculum. This reduces the possibility of healthy adults contracting virus disease from breeding sites and diminishes the perpetuation of this important biocontrol agent.

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