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# Preparation of a baculovirus inoculum for use by coconut farmers to control rhinoceros beetle *(Oryctes rhinoceros)*

B. Zelazny, A.R. Alfiler and A.M. Crawford

**Summary.** Crawford and Sheehan have continued their previous (1984) efforts to develop a baculovirus inoculum suitable for use by coconut farmers and extension workers in controlling rhinoceros beetles. The described inoculum can be prepared in a moderately equipped laboratory and is distributed in sealed vials. It can be stored at room temperature for several weeks, is stable in a refrigerator, and requires only simple procedures for infecting and releasing adult rhinoceros beetles. The method is currently being used by the Philippine Coconut Authority.

The rhinoceros beetle, *Oryctes rhinoceros* L., is a serious pest of coconut and oil-palm in Southeast Asia. Outbreaks often develop during replanting, when beetles breed in the trunks of felled palms and adult beetles attack and frequently kill young palms. Older palms are less vulnerable, but beetle damage causes loss of coconut yield (Young, 1975; Zelazny, 1979).

Control has concentrated on cultural methods, and on the use of a baculovirus disease of the beetle that was discovered in 1963 in Malaysia (Huger, 1966). The introduction of the virus into several South Pacific islands has effectively suppressed beetle populations there (Young, 1974; Bedford, 1981). In countries where the virus has become established or occurs naturally, efforts have begun to incorporate its use into integrated control programmes against the rhinoceros beetle (Zelazny, Alfiler and Mohamed, 1985; Zelazny and Alfiler, 1986).

As part of such control programmes, the release of virus-infected rhinoceros beetles appears promising, especially if the incidence of the disease is low in the outbreak area (Zelazny, 1977a, 1977b, 1984; Marschall and Ioane, 1982). As previous methods for infecting beetles required considerable experience in order to prepare virus inoculum, the aim

The work was done at the Philippines Coconut Authority (PCA) Albay Research Center, Guinobatan, Albay 4908, the Philippines, as part of the UNDP/FAO project "Coconut Pest and Diseases". B. Zelazny is currently with the UNDP/FAO project "Development of Integrated Pest Control", c/o FAO, PO box 2338, Jakarta, Indonesia. A.R. Alfiler is entomologist at the PCA Albay Research Center, and A.M. Crawford is with the New Zealand Department of Scientific and Industrial Research (DSIR), Entomology Division, Private Bag, Auckland, New Zealand.

**PRÉPARATION D'UN INOCULUM DE BACULOVIRUS A L'INTENTION DES PRODUCTEURS DE NOIX DE COCO POUR LUTTER CONTRE LE RHINOCÉROS *(ORYCTES RHINOCEROS)***

Résumé

**Crawford et Sheehan ont poursuivi leurs travaux précédents (1984) pour mettre au point un inoculum de baculovirus qui pourrait être utilisé par les producteurs de noix de coco et les vulgarisateurs pour lutter contre l'orycte rhinocéros. L'inoculum décrit peut être préparé dans un laboratoire moyennement équipé et il est distribué en flacons scellés, il est stable au réfrigérateur, et les méthodes utilisées pour infecter et relâcher les sujets adultes sont relativement simples. Cette forme de lutte est actuellement pratiquée par la Philippine Coconut Authority.**

**PREPARACION DE UN INOCULO DE BACULOVIRUS PARA USO DE LOS CULTIVADORES DE COCO EN SU LUCHA CONTRA EL ESCARABAJO RINOCERONTE *(ORYCTES RHINOCEROS)***

Resumen

**Crawford y Sheehan han proseguido su anterior (1984) actividad para conseguir un inóculo de baculovirus que puedan utilizar los cultivadores de coco y los extensionistas en la lucha contra los escarabajos rinocerontes. El inóculo descrito puede prepararse en un laboratorio dotado de un equipo de tipo medio, y se distribuye en viales herméticamente cerrados. Puede almacenarse a temperatura ambiente durante varias semanas, es estable en refrigerador y el procedimiento de infección y liberación de escarabajos rinocerontes adultos es sencillo. La Junta Filipina del Coco está utilizando este método en la actualidad.**

of the present study was to find a simple method that would allow coconut farmers and extension workers to infect and release beetles after only minimal training. Since the baculovirus inocula now used become inactivated within a few days at ambient temperature (Zelazny, 1972), a virus inoculum was needed that was easy to use, store and transport.

Crawford and Sheehan (1984) made first progress on this problem by demonstrating that when *O. rhinoceros* baculovirus is multiplied in *Heteronychus arator* cell cultures, the cell culture fluid demonstrates many of these desired properties. It remains infectious to other cell cultures for surprisingly long periods, even when stored at room temperature. As sugar can be added without affecting the shelf-life of the preparation, coconut farmers could infect rhinoceros beetles simply by placing a small drop of the cell culture fluid on the beetles' mouths. Marschall and Ioane (1982) found that rhinoceros beetles readily suck up sugar-virus suspensions.

In the present study, virus inoculum derived from *H. arator* cell cultures was tested on adult rhinoceros beetles in the Philippines. This method was compared with an earlier-described procedure for infecting beetles (Zelazny, 1978). Since the use of *H. arator* cell cultures is expensive and requires skill and experience not often available in countries with *O. rhinoceros* problems, other ways of preserving baculovirus infectivity were also investigated. Virus inocula prepared from guts of virus-infected *O. rhinoceros* adults were tested; these were preserved either by sterile filtration or with glycerol.

## Material and methods

*Preparation of virus inocula.* The *Heteronychus arator* cell culture was maintained and infected with the baculovirus according to the method described by Crawford (1982) and Crawford and Sheehan (1984). Virus was prepared from *O. rhinoceros* guts by glass permeation chromatography (Zelazny, Alfiler and Mohamed, 1985). Briefly, beetles were dissected two weeks after inoculation. Guts with clear signs of virus infections (white and swollen) were removed from five beetles and placed immediately on ice. Using a Pyrex # 7726 hand tissue grinder, they were then thoroughly homogenized on ice and suspended in 2 ml of a buffer containing 50 m*M* Tris-HCl (pH 8.5), 100 m*M* NaCl, 1 m*M* EDTA and 10 m*M* $Na_2SO_3$. The resulting suspension was clarified with a desk-top centrifuge for ten minutes at about 5 000 × *g*.

The supernatant was removed and placed on a 15-mm-wide chromatography column packed with controlled-pore glass (Sigma, 700 Å pore size, 120-200 mesh size) to a height of 80 cm (see Fig. 1). Before packing, the controlled-pore glass had been carefully washed in Tris-HCl buffer and deaerated under vacuum. The virus suspension was eluted from the column with 50 m*M* Tris- HCl buffer (pH 7.5) at a rate of about 1 ml per minute (see Fig. 2). The eluates were collected in a measuring cylinder and the first 60 ml of clear buffer were discarded. The next 20 ml were turbid and contained most of the virus. At least an

**Figure 1.** *Infecting a rhinoceros beetle by placing a drop of virus-containing culture fluid on its mouth*

additional 200 ml of buffer were passed through the column to wash out the bulk of soluble contaminants. In this way, the column could be used for one more purification cycle before requiring cleaning.

To clean the column, 150 ml of 1N HCl was passed through it, followed by about 250 ml of distilled water and at least 250 ml of buffer. The packed column was stored while containing 1N HCl, and could thus be used repeatedly provided that drying out was avoided. Occasionally, the top 2-3 cm of controlled-pore glass had to be replaced as they became contaminated with tissue debris.

Sucrose was added to the virus-containing fraction of the column eluate until it reached a concentration of 10 percent w/v. The preparation was pure enough to enable sterile filtration, which was performed inside a laminar flow cabinet. The virus preparation was forced through a 0.22-µm Milex disposable membrane filter (Millipore) using a large syringe. Up to 10 ml of eluate could be passed through each 25-mm-diameter filter. The filtrate was dispensed in 2-ml lots into sterile serum vials that were sealed with sterile rubber stoppers and crimped aluminium caps.

The vials were kept for one week at room temperature; those vials showing fungus growth or clouding due to bacterial contaminations were discarded. For glycerol preparations, virus-infected beetle guts were homogenized in concentrated glycerol, using 1 ml per gram of gut tissue.

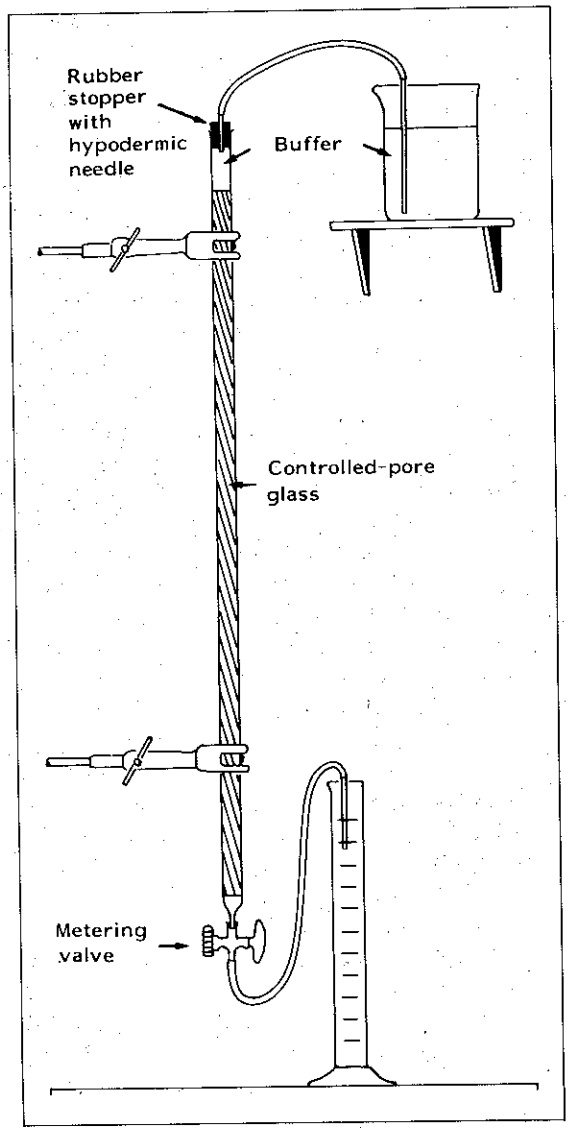


**Figure 2.** *Apparatus for partial purification of baculovirus from rhinoceros beetle guts with glass permeation chromatography. For loading the clarified gut homogenate, the rubber stopper is removed and the buffer is allowed to drain until it has reached the level of the controlled-pore glass. The gut extract is then loaded; as soon as it has been absorbed by the controlled-pore glass, buffer is added again. After replacing the stopper, the flow rate is adjusted to about 1 ml per minute. With a 15- × 800-mm column, most virus is eluted in the 60 to 80 ml fraction*

*Comparison of methods of infecting rhinoceros beetles.* Beetles used in the tests were reared from eggs or field-collected larvae on a 1:1 mixture of dried cow dung and decaying sawdust. The method of infecting *O. rhinoceros* adults by submerging them in a suspension of virus-killed larvae (Zelazny, 1978) has been practised in the Philippines by some government departments and commercial companies. Mass-reared rhinoceros beetles and virus inoculum from cell cultures were supplied to three such institutions. Workers were requested to infect about 50 beetles, either by placing a small drop of the supplied infectious cell culture fluid on the beetles' mouths (see Fig. 2) or by submerging them in virus suspensions, using their own stock of virus-killed larvae. Fifty beetles were kept uninfected as controls.

After inoculation, the beetles were kept individually in tins in moist sawdust for two weeks. They were then killed and their guts examined under a dissecting microscope for symptoms of baculovirus infection. In the case that no or doubtful symptoms were observed, a bioassay test using healthy *O. rhinoceros* larvae was conducted (Zelazny, 1978).

*Longevity of virus inocula.* The inocula were either stored at room temperature (average 28°C) or refrigerated at 4°C. Infectivity tests were conducted with fresh virus preparations and after storage for 1, 2, 5, 10, and 20 weeks by placing 10 µl on the mouth of each beetle. After the beetle sucked up the fluid, another drop of pure sucrose solution was fed to rinse off any virus remaining on the mouth parts. Concentrated preparations (but not the concentrated glycerol mixtures) were tested, as well as 10-, 100- and 1 000-fold dilutions in 10 percent sucrose.

Ten beetles per concentration were inoculated, and each dilution series included ten uninfected controls. Beetles were kept individually for two weeks and then diagnosed as above. The trials were usually replicated three times, although in a few cases only twice.

## Results

Table 1 shows that at two of the three institutions, submerging the beetles in a suspension of virus-killed larvae resulted in a very low rate of infection. This was presumably due to poor-quality inoculum; the virus-killed larvae may have been stored improperly or may have contained a large portion of larvae that died from other causes. Inoculating beetles by placing a small drop of virus-containing cell culture fluid on their mouth produced infections more consistently, although the response was still not quite as high as desired.

Similar results were obtained with the cell culture fluid in the laboratory trials. The sterile virus filtrate derived from the guts of infected beetles usually gave a higher rate of infection than the cell culture fluid, but it appeared to be slightly less stable during storage (see Table 2). The glycerol-virus mixture showed a high virulence when fresh but obviously could not be stored, even in a refrigerator.

TABLE 1. **Percentage of virus infections among *Oryctes rhinoceros* adults inoculated in two different ways at three institutions in the Philippines**

| Institutions | Submersion in suspension of virus-killed larvae | | Feeding with virus-containing cell culture fluid | | Uninfected control group | |
|---|---|---|---|---|---|---|
| | No. inoculated | % infected | No. inoculated | % infected | No. inoculated | % infected |
| Albay Research Center | 54 | 24 | 46 | 76 | 60 | 0 |
| Davao Research Center | 50 | 28 | 52 | 69 | 42 | 0 |
| Coco Investors Inc. | 48 | 88 | 49 | 63 | 49 | 0 |

The results in Table 2 seem to suggest that there was an upper limit in the percentage of infections which could not be surpassed, even after substantially increasing the virus concentration of the inoculum. This was most obvious from the responses obtained with the virus filtrate stored at 4°C. The reason for this was unclear, but may have been connected with the physiological state of the beetles or the inoculation procedure (e.g. a certain percentage of beetles might not have ingested any fluid). The results can best be analysed by assuming that about 20 percent of the beetles were "immune" to infection and then by following corresponding procedures of probit analysis (Finney, 1971). The regression lines giving the best fit for the combined responses in the trials with refrigerated inocula are shown in Figure 3. They indicate that the virus filtrate contained about 100 times as many infectious units as the cell culture fluid.

No substantial decrease in infectivity was apparent in the cell culture fluid or the sterile filtrate when refrigerated for up to 20 weeks or stored at room temperature under tropical conditions for up to two weeks (see Table 2).

TABLE 2. **Percentage of infections obtained in *O. rhinoceros* adults with three baculovirus preparations stored for various periods at room temperature or at 4°C**

| Type of inoculum | Concentration (1.0 = undiluted) | % infection | | | | | |
|---|---|---|---|---|---|---|---|
| | | Storage period in weeks | | | | | |
| | | 0 | 1 | 2 | 5 | 10 | 20 |
| *Stored at room temperature (average 28°C)* | | | | | | | |
| Cell culture fluid | 1.0 | 73 | 57 | 73 | 40 | 43 | 30 |
| | 0.1 | 57 | 47 | 73 | 33 | 30 | 33 |
| | 0.01 | 30 | 30 | 40 | 23 | 7 | 33 |
| | 0.001 | 13 | 7 | 13 | 10 | 10 | 7 |
| | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Sterile virus filtrate | 1.0 | 83 | 87 | 83 | 50 | 33 | 35* |
| | 0.1 | 97 | 83 | 83 | 57 | 57 | 20* |
| | 0.01 | 90 | 80 | 67 | 53 | 40 | 20* |
| | 0.001 | 60 | 63 | 40 | 27 | 20 | 15* |
| | 0 | 0 | 0 | 0 | 0 | 0 | 5* |
| Glycerol virus mixture | 0.1 | 97 | 0 | 0* | — | — | — |
| | 0.01 | 93 | 0 | 0* | — | — | — |
| | 0.001 | 80 | 0 | 0* | — | — | — |
| | 0 | 0 | 0 | 0* | — | — | — |
| *Stored at about 4°C* | | | | | | | |
| Cell culture fluid | 1.0 | | — | 77 | 70 | 50 | 70 |
| | 0.1 | | — | 73 | 23 | 43 | 53 |
| | 0.01 | | — | 50 | 30 | 40 | 30 |
| | 0.001 | | — | 13 | 7 | 10 | 17 |
| | 0 | | — | 0 | 0 | 0 | 0 |
| Sterile virus filtrate | 1.0 | | — | 90 | 73 | 73 | 73 |
| | 0.1 | | — | 83 | 80 | 77 | 73 |
| | 0.01 | | — | 93 | 83 | 60 | 77 |
| | 0.001 | | — | 80 | 47 | 37 | 40 |
| | 0 | | — | 0 | 0 | 0 | 3 |
| Glycerol virus mixture | 0.1 | | 50* | 90* | 10* | 0* | — |
| | 0.01 | | 55* | 40* | 10* | 0* | — |
| | 0.001 | | 45* | 5* | 0* | 0* | — |
| | 0 | | 0* | 0* | 0* | 0* | — |

NOTE: N = 30, *N = 20.

## Discussion

The infection of rhinoceros beetles with virus inoculum stored in sealed, sterile vials has distinct advantages over previous procedures. A stock of the vials can be kept in a refrigerator for ready use, and no experience in preparing the inoculum is required. The danger of using ineffective inoculum, resulting in the release of healthy beetles, can thus be greatly reduced.

The sealed vials also enable widespread use of the method by coconut farmers and extension workers. Transport, even to remote areas under tropical conditions, poses no problem, as two weeks' storage at room temperature did not result in any loss of infectivity. Nevertheless, direct exposure to sunlight or excessive heat must be avoided.

The use of the inoculum requires no special training; only a brief explanation and demonstration is necessary. The only equipment required is a supply of vials and medicinal droppers. To increase the infection rate, farmers should be instructed to place a second drop of inoculum on the mouth of each beetle

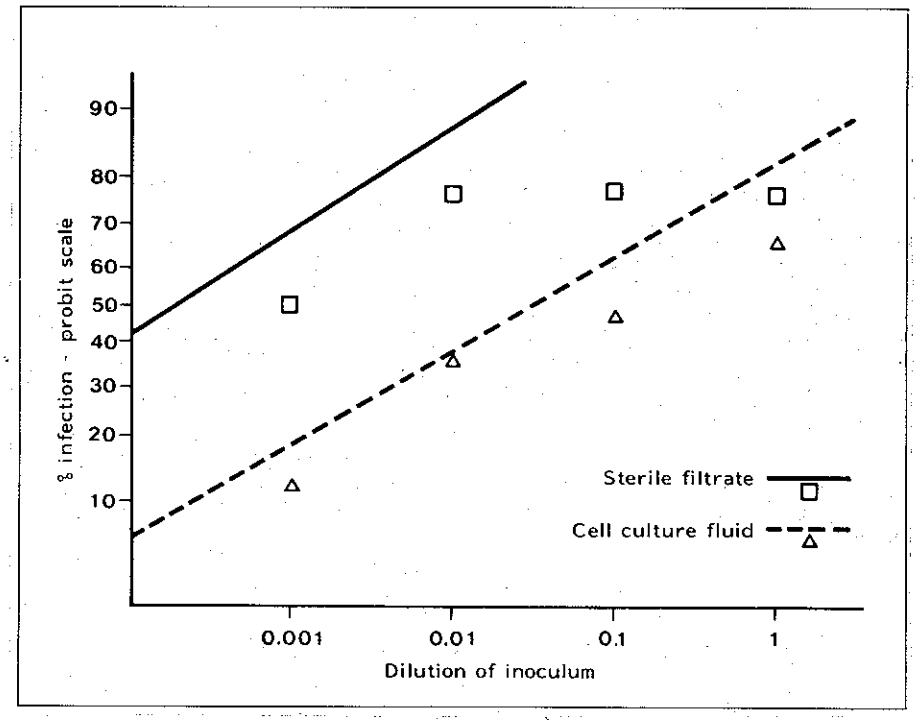


**Figure 3.** *Dosage-response correlations for infecting rhinoceros beetles with sterile virus filtrate or virus-containing cell culture fluid. The data are average infection rates obtained with inocula stored at 4°C for either 2, 5, 10 or 20 weeks. The straight lines are dosage/probit regressions fitted after assuming that 20 percent of the beetles were "immune" to infection*

after the first drop has been sucked up. The beetles are released by letting them fly out of a plastic basin just after dark. The basin should contain a large stone on which the beetles can climb for easier take-off.

Although the cost effectiveness of collecting beetles for virus releases remains to be determined, beetles are nevertheless at present routinely collected on many coconut and oil-palm plantations. It is clear that instead of killing these beetles, it would be far more beneficial to infect them with the virus and re-release them. This would involve very little additional effort and cost. Released infected beetles could no longer attack coconut palms or lay eggs (Zelazny, 1973, 1977a), and would spread the infection to other beetles and larvae.

The sterile virus filtrate described in this paper has a number of advantages over cell culture fluid. Its production involves less cost, skill and experience, and results in a higher virus concentration, which translates into a higher infection rate. Preparation of the filtered virus inoculum can be achieved in an only moderately equipped laboratory.

The average rate of infection was not higher in undiluted than in 1:10 diluted virus filtrate. Therefore, it may safely be recommended that the filtrate be diluted tenfold with 10 percent sucrose/Tris-HCL buffer before being dispensed into vials. This will result in a yield of 100 vials from five beetle guts at a negligible cost per vial. In fact, the main expense is the vials themselves; at present, the Philippine Coconut Authority, which is distributing inoculum in that country, requests return of the vials after use.

It would be desirable to conduct further trials to understand why a certain percentage of beetles did not become infected, even with high virus concentrations, and how to improve the infection rate. In addition, it might be possible to increase the stability of the virus filtrate by adding suitable substances such as soluble proteins. The cell culture fluid has a high protein concentration, which could be the reason for its slightly higher stability.

## References


BEDFORD, G.O. 1981. Control of the rhinoceros beetle by baculovirus. *In* Burges, H.D., ed. *Microbial control of pests and plant diseases*. London, Academic Press, pp. 409-426.

CRAWFORD, A.M. 1982. A coleopteran cell line derived from *Heteronychus arator* (Coleoptera: Scarabaeidae). *In Vitro*, 18: 813-816.

CRAWFORD, A. M. & SHEEHAN, C. 1984. An *Oryctes rhinoceros* (L.) (Coleoptera: Scarabaeidae) baculovirus inoculum derived from tissue culture. *J. Econ. Entomol.*, 77; 1610-1611.

FINNEY, D.J. 1971. *Probit analysis*. 3rd ed. Cambridge, UK, Cambridge Univ. Press.

HUGER, A.M. 1966. A virus disease of the Indian rhinoceros beetle *Oryctes rhinoceros* (Linnaeus), caused by a new type of insect virus, *Rhabdionvirus oryctes* gen. n., sp. n. *J. Invertebr. Pathol.*, 8: 38-51.

MARSCHALL, K.J. & IOANE, I. 1982. The effect of re-release of baculovirus in the biological control of rhinoceros beetles in Western Samoa. *J. Invertebr. Pathol.*, 39: 267-276.

YOUNG, E.C. 1974. The epizootiology of two pathogens of the coconut palm rhinoceros beetle. *J. Invertebr. Pathol.*, 24: 82-92.

YOUNG, E.C. 1975. *A study of rhinoceros beetle damage in coconut palms*. Noumea, New Caledonia, South Pacific Commission. Technical Paper No. 170.

ZELAZNY, B. 1972. Studies on *Rhabdionvirus oryctes*. I. Effect on larvae of *Oryctes rhinoceros* and inactivation of the virus. *J. Invertebr. Pathol.*, 20: 235-241.

ZELAZNY, B. 1973. Studies on *Rhabdionvirus oryctes*. II. Effect on adults of *Oryctes rhinoceros*. *J. Invertebr. Pathol.*, 22: 122-126.

ZELAZNY, B. 1977a. *Oryctes rhinoceros* populations and behaviour influenced by a baculovirus. *J. Invertebr. Pathol.*, 29: 210-215.

ZELAZNY, B. 1977b. Occurrence of the baculovirus disease of the coconut palm rhinoceros beetle in the Philippines and in Indonesia. *FAO Plant Prot. Bull.*, 25(2): 73-77.

ZELAZNY, B. 1978. Methods of inoculating and diagnosing the baculovirus disease of *Oryctes rhinoceros*. *FAO Plant Prot. Bull.*, 26(4): 163-168.

ZELAZNY, B. 1979. Loss in coconut yield due to *Oryctes rhinoceros* damage. *FAO Plant Prot. Bull.*, 27(3): 65-70.

ZELAZNY, B. 1984. Rhinoceros beetle *(Oryctes rhinoceros)* control on Bugsuk Island (Palawan). Report of the UNDP/FAO project "Coconut Pest and Diseases". Manila, 7 pp. (Mimeo)

ZELAZNY, B. & ALFILER, A. 1986. *Oryctes rhinoceros* (Coleoptera: Scarabaeidae) larva abundance and mortality factors in the Philippines. *Environ. Entomol.*, 15: 84-87.

ZELAZNY, B., ALFILER, A. & MOHAMED, N.A. 1985. Glass permeation chromatography for purification of the baculovirus of *Oryctes rhinoceros* (Coleoptera: Scarabaeidae). *J. Econ. Entomol.*, 78: 992-994.

ZELAZNY, B., CHIARAPPA, L. & KENMORE, P. 1985. Integrated pest control in developing countries. *FAO Plant Prot. Bull.*, 33(4): 147-158.