Asian Journal of Agricultural and Horticultural Research

2(2): 1-10, 2018; Article no.AJAHR.44385
ISSN: 2581-4478

Virulence of a New Isolate of Cytoplasmic Polyhedrosis Virus against the Red Palm Weevil, *Rhynchophorus ferrugineus* (Oliv.) (Order: Coleoptera, Family: Curculionidae)

Y. A. Mahmoud[1*], H. S. Salama[1], S. M. Moawed[1], I. M. A. Ebadah[1], H. E. Sadek[1] and I. A. Khalifa[2]

[1]Department of Pests and Plant Protection, National Research Center, Egypt.
[2]Plant Protection Institute, Agriculture Research Center, Egypt.

Authors' contribution

This work was carried out in collaboration between all authors. Authors YAM and HSS designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors SMM and IMAE managed the analyses of the study. Authors HES and IAK managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AJAHR/2018/44385
Editor(s):
(1) Dr. Ahmed Medhat Mohamed Al-Naggar, Professor of Plant Breeding, Department of Agronomy, Faculty of Agriculture, Cairo University, Egypt.
Reviewers:
(1) I. Merlin Kamala, Tamil Nadu Agricultural University, India.
(2) Parmeshwar Sahu, Indira Gandhi Agriculture University, India.
(3) R. P. Soundararajan, Tamil Nadu Agricultural University, India.
(4) Isela Quintero Zapata, Universidad Autonóma de Nuevo León, Mexico.
Complete Peer review History: http://prh.sdiarticle3.com/review-history/26941

Original Research Article

Received 12 July 2018
Accepted 28 September 2018
Published 31 October 2018

ABSTRACT

The red palm weevil (RPW), *Rhynchophorus ferrugineus* (Oliv.) is a serious pest of date palms and causes substantial losses worldwide. Due to its hidden nature of habitat, managing this pest is extremely difficult. This study reveals a new biological control agent that is beneficial for controlling this pest. In the laboratory, the virus was isolated from a colony of diseased larvae; the median lethal dose (LD_{50}) and median lethal time (LT_{50}) of this virus were determined, and the biological activities of the virus were assessed. Microscopic examination of the diseased larvae provided an evidence of the presence of polyhedral inclusion bodies (PIBs) in all typical tissues where the virus is known to develop. Electron microscopic examination revealed various shapes of polyhedra,

**Corresponding author: Email: youssefmahamoud@yahoo.com;*

most of which were hexagonal and tetragonal. Experiments were conducted to investigate viral propagation and virulence against different larval instars. The results showed that the initial infected dose (5×10^3 PIB/larva) increased to 600×10^9 PIB/larva. The LT_{50} values decreased as both viral concentration and larval age increased. Moreover, the percentage of larval mortality significantly increased as larval age increased. It can be concluded that a viral dose of 80×10^6 PIB/ml is sufficient and adequate for suppressing weevil population.

Keywords: Date palm pest; larval mortality; biological control.

1. INTRODUCTION

Since 1992, the red palm weevil (RPW), *Rhynchophorus ferrugineus* (Oliv.) has become one of the most damaging pests of palm trees in the northern region of Egypt and damage from this pest can lead to severe losses [1,2].

Many efforts have been made to control this pest using different tools, such as chemical insecticides [3-7], pheromones [8-13] and gamma radiation [14,15]. Other biocontrol agents, including yeast [16,17]; bacteria, *Pseudomonas aeruginosa* [18-20]; fungi [21-29] and cytoplasmic polyhedrosis virus (CPV) [30-33] have also been used. Furthermore, reared colonies of this pest, especially those reared in the laboratory; appear to be susceptible to several entomopathogens [34]. We hope that this study will help researchers uncover critical unexplored areas of controlling this serious pest.

This study involved laboratory investigations of a disease caused by a virus originally isolated from infected and dead RPW larvae. Experiments were carried out on the purification, propagation, and assessment of biological activities of this virus. The dose - mortality (median lethal dose, LD_{50}) and time – mortality (median lethal time, LT_{50}) were subsequently determined.

2. MATERIALS AND METHODS

2.1 Rearing Technique

The methods of Salama, Moawed, 2008 were adopted. RPW larvae were obtained from the standard colony at the pests and plant protection laboratory of National Research Center and maintained at 25 ± 2 ^{0}C and 70 – 80% relative humidity (RH). Samples of larvae, pupae and adults were collected from the colony, distributed into plastic trays (30×20×10 cm) that had perforated covers, and reared on banana or sugar cane. The adults were distributed into similar plastic trays at a ratio of five males: ten females per tray and supplied with sugar cane pieces which served as an oviposition site and as a food source.

2.2 Isolation, Purification, and Identification of Polyhedral

The principal method of Bergold [35] regarding the isolation and purification of viruses within spruce budworm, *Choristoneura fumiferana* was adopted. The diseased larvae and cadavers exhibiting symptoms were macerated, suspended in water, blended and filtered through a muslin cloth to remove large debris. The aqueous suspension containing the polyhedral inclusion bodies (PIBs) was subsequently purified by repeated centrifugation at 4000 rpm for ten minutes. The collected polyhedra were then washed with distilled water, and centrifugation was repeated; the polyhedra were the maintained in the refrigerator at 4ºC as an aqueous suspension ready for use. Further confirmation was achieved by reinfection of 30 - day- old larvae and re isolation of the polyhedra from those cadavers.

Identification of highly purified polyhedra was performed by using an electron microscope. The method of Vander Gest [36], as modified by many authors was used [37-39]. A stock suspension was standardized by using a hemocytometer (Burker chamber) [34].

2.3 Artificial Infection

The virus was incorporated into the larval diet [39]. Four viral concentrations were prepared: 10×10^6, 20×10^6, 40×10^6 and 80×10^6PIB/ml. One milliliter of each concentration was removed by a small pipette, and half of this dose (0.5 ml) was distributed onto the upper surface and the sides of a piece of banana fruit (100 gm), after which several holes were created on the upper surface of the piece by using a fine needle. The second half of the dose was distributed onto the perforated piece and administered to the larvae.

2.4 Rate of Propagation of Ingested Virus Dose

Bioassays were carried out using the droplet - feeding method [40]. Larvae were allowed to sip 1µl from the virus suspension (5×10^6 PIB/ml = 5×10^3 PIB per larva). Before sipping, the larvae starved for 24 hours, and 30 larvae (25 days old) were infected, after which the larvae were transferred to separate cups and maintained at 25ºC. Larval mortality was recorded daily. Dead larvae were collected, and the virus was isolated from only one dead larva; the polyhedra were maintained in 100 ml of distilled water. Five suspensions, which served as replications of five dead larvae, were used for counting polyhedra.

2.5 Bioassay Technique

Fifteen-, 30- and 45 - day - old larvae were placed into plastic cups (10- cm diameter and 7-cm height) that had perforated covers for ventilation; the larvae were then allowed to feed on a diet contaminated with the virus (10 g per larva) as previously mentioned. Seventy- five larvae were used for each concentration. A similar number of larvae fed on diet treated with 1 ml of sterile distilled water; these larvae served as a check. After consuming all the treated diet, the larvae fed on untreated fresh food. The larvae were examined daily until each larva had either died or pupated.

The samples of dead larvae or pupae were examined for the presence of the pathogen under a phase - contrast microscope. All experiments were carried out at 25±2^{o}C and 70±5% RH. Percent mortality was calculated, and the insects that failed to survive to adulthood were considered dead.

2.6 Statistical Analysis

The results were subjected to probit analysis in accordance with the Finney method [41], and the data were statistically analyzed using F-tests (ANOVA) to compare the LD50 values. Duncan's multiple range test was used to compare mortalities [42].

3. RESULTS AND DISCUSSION

3.1 Symptomatology of the Disease

The administration of the viral concentrations to the RPW larvae (15-30 days old) led to the development of typical viral symptoms that clearly appeared on the older larvae. The observed symptoms could be described as follows: the normal white color changed to a yellowish color 7-10 days after infection. The larvae whose symptoms appeared late consumed less food. The diseased larvae fed always on the external surfaces of the sugar cane pieces, while the healthy larvae fed on the interior parts. The larvae became swollen and lethargic, and the body became flabby after which death occurred (Fig. 1). When the older larvae (30-45 days old) were infected with the virus, some died at the larval stage; and the others died as pupae or emerge later as deformed adults. Most of the treated larvae died in their cocoons leading to a deformed larval-pupal stage.

These symptoms of the diseased RPW larvae somewhat agree with those caused by other baculoviruses in their respective hosts [43-49]. The presence of an intermediate stage (larval - pupal stage) that developed from infected larvae may be due to an increase in juvenile hormone in the diseased insects as a result of infection [50,51].

3.2 Polyhedra

Many polyhedra were suspended in the body fluids in the samples of dead larvae examined under the light microscope. Additionally, numerous polyhedra were observed in the fat bodies, which appeared as spherical bodies measuring 0.3µm in diameter (Fig. 2).

Electron microscopic examination of the highly purified polyhedra revealed that they are irregular in shape and vary in diameter (0.3 - 7µm). The majority are polygonal (tetra - hexagonal) and have a diameter of 1.5 - 2.5µm or have navicular or spherical (5 - 7µm) shape (Fig. 3).

These measurements and descriptions are similar to those mentioned by some authors [34,52] and regarding insect virus classification. The description of this virus agrees with that of other viruses infecting coleopteran insects such as the Indian rhinoceros beetle, *Oryctes rhinoceros* (Linnaeus) [53-55], and the RPW of coconut [30].

3.3 Rate of Virus Propagation

The virus was propagated by infecting 25 – day-old larva by allowing the larvae to sip 1 µl of the viral suspension that contained 5×10^6 PIB/ml

(equal to = 5×10^3 PIB per larva). Table 1 shows the relationship between the amount of input and output of the polyhedra. The initial ingested dose (5×10^3 PIB per larva) clearly increased to 3×10^9 PIB per larva; this means that the rate of virus propagation was 1: 6×10^3 polyhedra at the end of infection or until the death of the larvae. The virus mass multiplied by 600.00 PIB from one PIB. By re-examining infections of the Egyptian cotton leaf worm, *Spodoptera littoralis* (Boisduval), by its native nuclear polyhedrosis virus (NPV), the initial dose (7500 PIB/larva) grew to 2.9×10^9 PIB/larva [39]. This finding means that one polyhedron multiplied and produced 380000 polyhedra during infection.

3.4 Effects on Larvae

The virulence of cytoplasmic polyhedrosis virus (CPV) on *R. ferrugineus* larvae was expressed as the percent mortality among 15-, 30-, and 45-days – old treated larvae. The results in Table 2 show that all tested viral concentrations caused higher larval mortality than the control. Percent mortality increased as viral concentrations increased, while susceptibility to the pathogen decreased as larval age increased at any given dose. For example, the viral concentration of 20×10^6 PIB/ml caused 89.3%, 73.4% and 58.8% mortality in the 15-, 30- and 45-day-old larvae, respectively.

3.5 Effects on Pupae and Adults (PA)

The pupae that resulted from post - infected larvae, failed to emerge or emerged as deformed adults were considered dead. Table 2 shows that the larvae at all ages gave similar responses to all tested concentrations. Percent pupal and adult mortalities ranged from 84 - 100%.

3.6 Cumulative Effect of CPV on Larvae, Pupae, and Adults (LPA)

The harmful effects as described by the percent mortality of LPA, were greater in younger larvae (15 - days old) than in older larvae (30- and 45 - day- old). The continuous presence of the virus in the pupae may explain the non-significant percent mortality differences among the LPA of all ages (Table 2).

3.7 Time - Mortality Data (LT_{50}) Due to CPV

The daily mortality data that were recorded, could be used to estimate could be made of the median lethal time (LT_{50}) for each larval age at given dose (Table 3). Only those larvae finally dying from the virus were included in the calculations.

The values of LT_{50} were decreased with increasing of both viral concentration and younger age. These values increased from 8.5 - 17.0 days for 15 - days old larvae to 10.5 - 22.0 days for 45 - days old larvae. With higher viral concentration (80×10^6 PIB/ml) LT_{50} values ranged between 8.5 - 10.5 days while there are no significant differences between the two oldest ones (30- and 45 - days old larvae).

3.8 Dose - Mortality Data (LD_{50})

The LD_{50} values and associated statistics of CPV against the three larval ages of *R. ferrugineus* are presented in Table 4. The LD_{50} increased from 6.7×10^6 PIB/ml for 15 - day- old larvae to 14.5×10^6 and 18.4×10^6 PIB/ml for 30- and 45-day- old larvae, respectively. There was a significant difference in LD_{50} values between the three larval ages. The older larvae (30-45 days) have LD_{50} values approximately 2.0 and 3.0 times greater, respectively, than those of 15-day- old larvae. The older larvae are much less susceptible, but the deleterious effects of the virus appeared in subsequent stages. Several studies have highlighted the potential benefits of using viruses as entomopathogens to maintain insect pests populations below economic thresholds. In the present study, the obtained results concerning larval, pupal and adult mortalities; LT_{50}; LD_{50}; and deleterious effects of the virus strongly agree with those previously reported on Western oak looper, *Lambdina fiscellaria somniaria* (Hulst) [56], Gypsy moth, *Lymantria dispar* (Linnaeus) [57], Egyptian cotton leaf worm, *S. littoralis* [39] and the black cutworm, *Agrotis ipsilon* (Hufnagel) [49] in that the LT_{50} values decreased as the viral dose increased. A similar phenomenon: high doses led to a rapid effect, while lower doses required more time to develop and, propagate, after which the effects appeared [58].

Additionally, an increase in mortality was generally accompanied by a decrease in the LT_{50} values at all tested concentrations, and the LD_{50} values increased as larval age increased. Similar conclusions were reached on the effects of baculoviruses on other harmful pests [59-67]. Decreased larval susceptibility was strongly correlated increased body weight. Moreover, infection with the CPV during all developmental stages of RPW was reported in India. In the

present study, the infected larvae emerged as deformed adults, and these deformations led to significant suppression of the weevil population.

Many possible reasons for differences in the progression of a disease in different instars have been suggested by Vail and Hall [68]: the effective dose per larva may vary according to (1) alkalinity of the mid gut required for dissolution of the ingested polyhedra and/or for the survival of viral particles, (2) the number of viral particles entering the hemocoel, and (3) the virulence of viral particles that enter the hemocoel.

Table 1. Rate of propagation of RFCPV and mean number of polyhedral presented in one infected larva

Replicate (a)	PIB / one big square (Mean ± SE)	No. PIB / Larva		Ratio (d) 1 : 2
		Input	Output	
1	13.6 ± 0.45			
2	13.2 ± 1.65			
3	10.2 ± 0.86	5×10^3	3×10^9 (c)	$1 : 600 \times 10^3$
4	16.4 ± 1.21			
5	11.0 ± 0.92			
General mean	12.0 ± 1.03 (b)			

(a) Five different suspensions prepared from 5 – dead larvae
(b) Number of PIB / one big square in the haemocytometer
No. PIB / µl = b × conc. × dilution
(c) No. PIB / µl = 12 × 250 ×10 = 30200 ~ 30000
No. PIB / ml = 30000 $\times 10^3$ = 30 $\times 10^6$
No. PIB / larva = 30 $\times 10^6$ x 100 (Total volume) = 3 $\times 10^9$
(d) Rate of propagation (Ratio) = input / output

Table 2. Percent mortality among immature stages at different ages of *R. ferrugienus* exposed to its RFCPV

Age (Days)	Viral conc. 1 $\times 10^6$ PIB / ml	% Mortalities *		
		L	PA	LPA
15	0	10.7 a	4.5 a	14.7 a
	10	77.4 b	88.2 c	97.3 b
	20	89.3 c	100.0 d	100.0 b
	40	89.3 c	75.0 b	97.3 b
	80	100.0 d	0.0	100.0 b
30	0	5.4 a	5.6 a	10.7 a
	10	48.0 b	87.2 b	93.3 b
	20	73.4 c	85.0 b	96.0 bc
	40	81.3 cd	92.9 bc	98.7 bc
	80	88.0 d	100.0 c	100.0 c
45	0	4.0 a	4.2 a	8.0 a
	10	39.9 b	87.8 bc	86.7 b
	20	58.8 c	88.1 bc	94.7 c
	40	74.7 d	84.2 b	96.0 c
	80	79.9 d	93.3 c	98.7 c

L = Larvae, PA = Pupae and Adults, LPA = Larvae, Pupae and Adults
** Values followed by the same letters are not significantly different (P > 0.05)*

Table 3. LT_{50} – values of *R. ferrugienus* larvae after infecting with different concentrations of its RFCPV

Larval age (days)	Viral concentration (PIB / ml)			
	10 x 10^6	20 x 10^5	40 x 10^6	80 x 10^6
15	17.0	10.0	11.5	8.5
30	0.0 *	15.5	13.5	10.5
45	0.0 *	22.0	12.0	10.5

** No data available and percent larval mortality was less than 50%*

Table 4. LD_{50} – values of *R. ferrugienus* larvae after infecting with different concentrations of its RFCPV

Larval age (days)	LD_{50} PIB / ml *	90 % Fiducial limit		Slope ± SE	Susceptibility level **
		lower	upper		
15	6.7×10^6 a	4.6	8.3	1.48 ± 0.306	1.0
30	14.5×10^6 b	11.3	18.6	2.20 ± 0.245	2.16
45	18.4×10^6 b	12.4	22.8	2.13 ± 0.173	2.75

** Values followed by the same letters are not significantly different (P > 0.05)*
*** Susceptibility level = LD_{50} of every larval age / LD_{50} of 15 day – larval age*

Fig. 1. Dead and intermediate larval – pupal stage of RPW larvae due to treatment with CPV

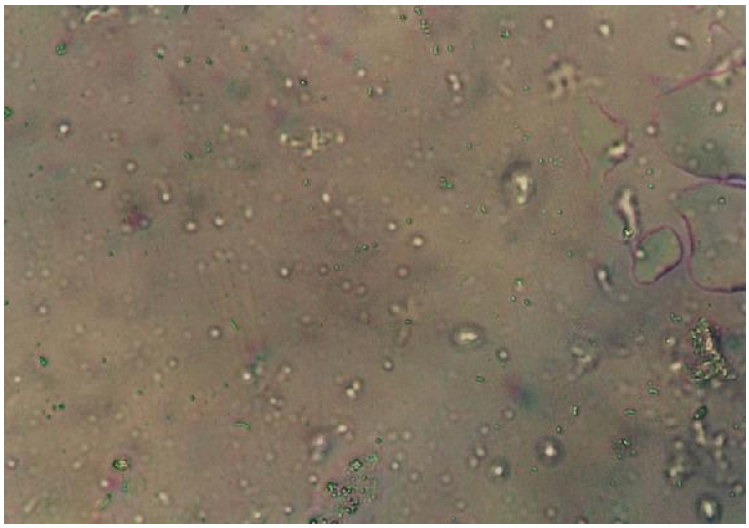

Fig. 2. Spherical white polyhedral (light microscope)

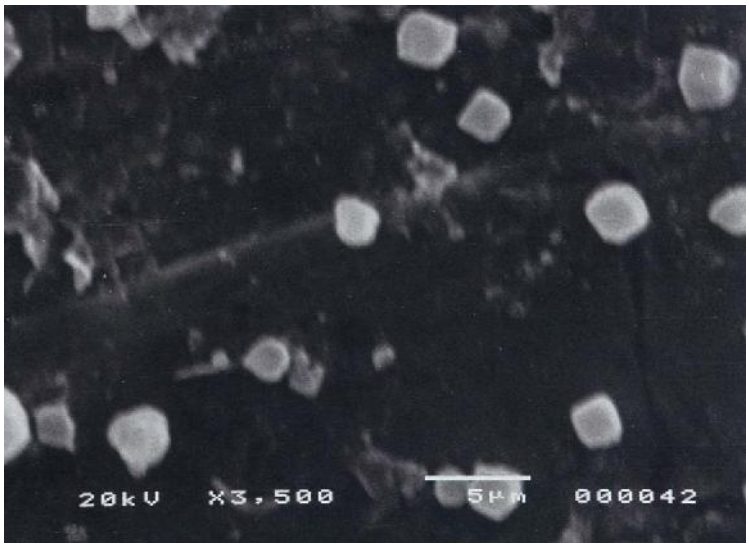

Fig. 3. Electron microscope (X3.500): Different shapes of polyhedra, Nanvicular, Spherical, Tetragonal and Hexagonal

4. CONCLUSION

The use of viruses obtained from naturally infected RPW, including CPV, should be seriously considered for biological control of this pest. A viral dose of 80×10^6 PIB/larva was sufficient and adequate for suppressing weevil populations; this dose resulted in 80 - 100% larval mortality. Always, in date palm plantations, the irrigation regime seems to affect RPW infestation. The continual contact of water at the stipe base creates a favorable environment for RPWs to lay their eggs. Additionally, severely damaged and dead palm trees should be eliminated.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Cox ML. Red palm weevil, *Rhynchophorus ferrogineus* in Egypt. FAO Plant Protection Bulliten. 1993;41:30-31.
2. El-Garhy ME. Field evaluation of the aggregation pheromones of the red palm weevil, *Rynchophorus ferrogineus* in Egypt. Brighton Crop Protection Conference on Pests and Diseases. 1996;3:1059-1064.
3. El-Ezaby F. A biological in vitro study on Indian date palm weevil. Arab Journal of Plant Protection. 1997;15:84-87.
4. Cabello T, Pena J, Barranco P, Belda J. Laboratory evaluation of imidacloprid and oxamyl against *Rhynchophorus ferrogineus.* Annals of Applied Biology, 130 Suppl Tests Agrochem Cultivars. 1997;18: 6-7.
5. Barranco P, Peña JA, De la Martin MM, Cabello T. Efficacy of chemical control of the new palm pest *Rhynchophorus ferrugineus* (Olivier) (Curculionidae: Coleoptera). Boletin de Sanidad Vegetal Plagas. 1998;24:301–306.
6. Llácer E, Dembilio Ó, Jacas JA. Evaluation of the efficacy of an insecticidal paint based on chlorpyrifos and pyriproxyfen in a microencapsulated formulation against *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae). Journal of Economic Entomology. 2010;103:402–408.
7. Dembilio Ó, Jaques JA. Biology and management of red palm Weevil. In: W. Wakil et al. (Eds.), Sustainable Pest Management in Date Palm: Current Status and Emerging Challenges, Sustainability in Plant and Crop Protection; 2015. DOI:10.1007/978-3-319-24397-9_2
8. Cork A, Hall DR. Application of pheromones for crop pest management in Indian sub-contient. Journal of Asia Pacific Entomology. 1998;1:35-49.
9. Falerio JR, Abraham VA, Al-Shuaibi MA. Role of pheromone trapping in the management of red palm weevil. Indian Coconut Journal of Cochina. 1998;29:1-3.

10. Abraham VA, Faleiro J R, Al Shuaibi M A, Al-Abdan S. Status of pheromone trap captured female red palm weevils from date gardens in Saudi Arabia. Journal of Tropical Agriculture. 2001;39:197–199.
11. Faleiro JR. Pheromone technology for the management of red palm weevil, *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Rhynchophoridae) – A key pest of coconut (Technical Bulletin No. 4). ICAR Research Complex, Goa. 2005;40.
12. Faleiro JR, Abo-El-Saad M, Al-Abbad AH. Pheromone trap density to mass trap *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae) in date plantations of Saudi Arabia. International Journal of Tropical Insect Science. 2011;31:75–77.
13. Abuagla AM, Al-Deeb MA. Effect of bait quantity and trap color on the trapping efficacy of the pheromone trap for the red palm weevil, *Rhynchophorus ferrugineus*. Journal of Insect Science. 2012;12:120. DOI:10.1673/031.012.12002.
14. Ramachandran CP. Effect of gamma radiation on various stages of red palm weevil *Rhyncophorus ferrugineus* F. Journal of Nuclear Agriculture and Biology. 1991;20:218-221.
15. Al-Aydeh HY, Rasool KG. Determination of the optimum sterilizing radiation dose for control of the red date palm weevil, *Rhynchophorus ferrugineus* Oliv. (Coleoptera: Curculionidae). Crop Protection. 2010;29:1377–1380.
16. Dangar TK. Infection of red palm weevil, *Rynchophorus ferrogineus* by yeast. Journal of Plantation Crop. 1997;25:193-196.
17. Salama HS, Foda MS, El-Bendary MA, Abdel-Razek A. Infection of red palm weevil, *Rhynchophorus ferrugineus*, by spore-forming bacilli indigenous to its natural habitat in Egypt. Journal of Pest Science. 2004;77:27-31.
18. Banerjec A, Dangar TK. Pseudomonas aeruginesa, a facultative pathogen of red palm weevil, *Rynchophorus ferrogineus*. World Journal of Microbiology and Biotechnology. 1995;11:618-620.
19. Francesca N, Caldarella CG, Moschetti G. Indagini preliminari su bacilli sporigeni associati ad adulti di Punteruolo rosso e loro possibili impieghi in lotta biologica. In: La ricerca scientifica sul punteruolo rosso e gli altri fitofagi delle palme in Sicilia, Regione Siciliana-Assessorato Agricoltura e Foreste Dipartimento Interventi Infrastrutturali, Servizi allo Sviluppo. 2008;I:69–72.
20. Francesca N, Alfonzo A, Verde GL, Settanni L, Sinacori M, Lucido P, Moschetti G. Biological activity of *Bacillus* spp. evaluated on eggs and larvae of red palm weevil *Rhynchophorus ferrugineus*. Annals of Microbiology. 2015;65:477–485.
21. Galani G, Andrei A. Effect of some formulation substances on germination and virulence of *Beauveria bassiana* and *Verticillium lecanii* conidia. IOBC/WPRS Bulliten. 1996;19:218-220.
22. Burgess HD. Formulations of myco-insectisides, pp. 131-185. In “Burges H.D. (Ed.), Formulation of microbial Biopesticides”. Kluwer Acd. Publications, Great Britin.
23. Feng MG, Poprawski TJ, Khachatourians GG. Production, formulation and application of entomopathogenic fungus, *Beauveria bassiana* for insect control. Current Status – Biocontrol science and Technology. 1998;4:3-34.
24. Gindin G, Levski S, Glazer I, Soroker V. Evaluation of the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* against the red palm weevil *Rhynchophorus ferrugineus*. Phytoparasitica. 2006;34:370–379.
25. El-Sufty R, Al-Awash SA, Al Bgham S, Shahdad AS, Al Bathra AH. Pathogenicity of the fungus *Beauveria bassiana* (Bals.) to the red palm weevil, *Rhynchophorus ferrugineus* (Oliv.) (Col.: Curculionidae) under laboratory and field conditions. Egyptian Journal of Biological Pest Control. 2009;19:81.
26. Dembilio Ó, Quesada-Moraga E, Santiago-Álvarezand C, Jacas JA. Potential of an indigenous strain of the entomopathogenic fungus *Beauveria bassiana* as a biological control agent against the red palm weevil, *Rhynchophorus ferrugineus*. Journal of Invertebrate Pathology. 2010;104:214–221.
27. Hajjar MJ, Ajlanand AM, Al-Ahmad MH. New approach of *Beauveria bassiana* to control the red palm weevil (Coleoptera: Curculionidae) by trapping technique. Journal of Economic Entomology. 2015;108:425–432.
28. Jalinas J, Güerri-Agulló B, Mankin RW, López-Follana R, Lopez-Llorca LV. Acoustic assessment of *Beauveria bassiana* (Hypocreales: Clavicipitaceae) effects on *Rhynchophorus ferrugineus*

(Coleoptera: Dryophthoridae) larval activity and mortality. Journal of Economic Entomology. 2015;108:444-453.

29. Lo Verde G, Torta L, Mondello V, Caldarella CG, Burruano S, Caleca V. Pathogenicity bioassays of isolates of *Beauveria bassiana* on *Rhynchophorus ferrugineus*. Pest Management Science. 2015;71:323–328.
30. Gopinadhan PB, Mohandas N, Nair KPV. Cytoplasmic polyhedrosis virus infecting red palm weevil of coconut. Current Science. 1990;59:577-580.
31. Alfazairy AA, Hendi R, El-Minshawy AM, Karam HH. Entomopathogenic agents isolated from 19 coleopteran insect pests in Egypt. Egyptian Journal of Biological Pest Control. 2003;13:233-238.
32. Alfazariy AA. Notes on the survival capacity of two naturally occurring entomopathogens on the red palm weevil *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae). Egyptian Journal of Biological Pest Control. 2004;14:423.
33. El-Minshawy AM, Hendi RA, Gadelhak GG. Viability of stored polyhedrosis virus on the red palm weevil, *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae). In: FAO/IAEA International Conference on Area-Wide Control of Insect Pests: Integrating the Sterile Insect and Related Nuclear and Other Techniques, Vienna, Austria. 2005;241–242.
34. Weiser J. An Atlas of Insect Diseases. 1977; 400 Fig + 240pp. Academic Science, Prague, Czechoslovakia.
35. Bergold GH. Die Isolierung des Polyheder virus und die Nature der Polyheder. Zeitschrift Naturfarsch. 1947;2b:122-143.
36. Vander-Geest LPS. A method for the purification of polyhedral bodies. Journal of Invertebrate Pathology. 1968;11:502.
37. Scott HA, Young SY, McMasters JA. Isolation and some properties of the components of nuclear polyhedral from the cabbage looper, *Trichplusia ni*. Journal of Invertebrate Pathology. 1971;18:177-182.
38. Monsarrat P, Veyrunes J, Meynadier G, Croizier G, Vago C. Purification et etude structurate du virus du coleopteran *Oryctes rhinoceros* L. C.R. Academic Science Paris. 1973;277:1413-1415.
39. Moawed SM. The nuclearpolyhedrosis virus of *Spodoptera littoralis* as biological insecticide: its virulence, pathogenicity, production and interference with other viruses and microsporidia. Ph D Thesis, Czecho, I. Academic Science, Prague. 1980;296.
40. Moawed SM, Salama HS, Foda M, Zaki FN. Potency of combinations of *Bacillus thuringiensis* and chemical insecticides vs. *Spodoptera littoralis*. Annals of Agricultural Science. 1984;59:83-88.
41. Finney DI. Probit analysis. 3rd ed. Cambridge Univ. Press, London; 1971.
42. Duncan DB. Multiple rang and multiple F-test. Biometrics. 1955;11:1-42.
43. Tanada Y, Chang GY. A cytoplasmic polyhrdrosis of the armyworm, *Pseudoletia unipucta* H., Lepidoptera; Noctuidae. Journal of Invertebrate Pathology. 1960;21:201-208.
44. Aizawa K. The nature of infection caused by nuclear polyhedrosis virus. In “Insect Pathology, an Advanced Treatise”. (E.A. Steinhaus, ed. 1963;1:381-412. Acad. Press, New York, London).
45. Smith KM. The cytoplasmic virus diseases. In “Insect Pathology an Advanced treatise”. C.E.A. Steinhaus, ed. 1963;1:457-97. Acad. Press, New York and London.
46. Martignoni ME. Progressive nucleopolyhedrosis in adults of *Peridroma soucia* H. Journal of Invertebrate Pathology. 1964;6:368-372.
47. Whitlock VH. Dosage-mortality studies of a granulosis and nuclear polyhedrosis viruses of a laboratory strain of *Heliothis armigera*. Journal of invertebrate Pathology. 1978;32:386-387.
48. Moawed SM, Saleh MR, Salem SA. Interaction of the microsporidian, *Vairimorpha epheatiae* with nuclear polyhedrosis virus and their effect on Egyptian cotton leafworm, *Spodoptera littoralis*. Acta Entomologica Bohemoslvanica. 1987;84:422-430.
49. Salama HS, Moawed SM. Joint action of nuclear polyhedrosis virus and chemical insecticides against the black cutworm, *Agrotis ipsilon* H. Acta Biologica Hungarica. 1988;39:99-107.
50. Fisher F, Sanborn R. Nosema as a source of Juvenile hormone in parasitized insects. Biological Bulletin. 1964;126:235-252.
51. Fowler JL, Reeves EL. In vivo propagation of a microsporadian pathogenic to insects. Journal of Invertebrate Pathology. 1975;25:349-353.
52. Wildly P. Classification and nomenclature of viruses. In “Monographs in Virology.”

J.L. Melnick (Ed.). 1971;5:17-32. New York.
53. Hugar AM. A virus disease of the Indian rhinoceros beetle *Oryctes rhinoceros* L. Journal of invertebrate Pathology. 1966;8: 38-51.
54. Bedford GO. Use of a virus against the coconut palm rhinoceras beetle in Fiji. Pest Articles News Summ (PANS). 1976;22: 11–25.
55. Pyne CC, Compson D, Looz SM. Properties of the nucleocapsids of a virus isolated from *Oryctes rhinoceros*. Virology. 1977;77:269-280.
56. Morris ON. Quantitative infectivity studies on the nuclear polyhedrosis virus of the western oak looper, *Lambdina fiscellaria* H. Journal of Insect Pathology. 1962;4:207-215.
57. Doane CC. Bioassay of nuclear polyhedrosis virus against larval instars of gypsy moth. Journal of Invertebrate Pathology. 1967;9:376-386.
58. Salama HS, Moawed SM. A method for the control of the red palm weevil *Rhynchophorus ferrugineus* (F) using Microsporidia. Egyptian Patent No. 24099/2008. 2008.
59. Allen GE, Ignoffo GM. The nuclear polyhedrosis virus of *Heliothis*. Quantative in vivo estimates of virulence. Journal of Invertebrate Pathology. 1969;13:378-381.
60. Chautani AR, Rehnborg GS. Doses mortality data on the nuclear polyhedrosis viruses of *Spodoptera exigua*, *Trichoplusia ni* and *Prodenia ornithognlli*. Journal of Invertebrate Pathology. 1971;17:234-237.
61. Klein M, Poddler H. Studies on the application of a nuclear polyhedrosis virus to control population of Egyptian cotton worm, *Spodoptera littoralis*. Journal of Invertebrate Pathology. 1987;32:244-248.
62. Evans HF. Quantative assessment of the relationship between dosage and response of the nuclear polyhedrosis virus of *Mamestra brassicae*. Journal of Invertebrate Pathology. 1981;37:101-109.
63. Evans HF. The influence of larval maturation on responses of *Mamestra brassicae* L. (Lepidoptera: Noctuidae) to nuclear polyhedrosis virus infection. Archives of Virology. 1983;75:163-170.
64. Hughes PR, Getting RR, McCarthy WJ. Comparison of the time-mortality response of *Heliothis zea* to 14 isolates of *Heliothis* nuclear polyhedrosis virus. Journal of Invertebrate Pathology. 1983;41:256-261.
65. Teakle RE, Jensen JM, Giles JE. Age-related susceptibility of *Heliothis punctiger* to a commercial formulation of nuclear polyhedrosis virus. Journal of Invertebrate Pathology. 1986;47:82-92.
66. Salam HS, Moawed SM, Megahed MI. Effect of nuclear polyhedrosis virus on the cotton bollworm, *Heliothis armigera* H. Zeitschrift Angewandte Entomologie. 1986;102:123-130.
67. Smits PH, Vlak JM. Biological activity of *Spodoptera exigua* nuclear polyhedrosis virus against *S. exigua* larvae. Journal of Invertebrate Pathology. 1988;51:107-114.
68. Vail PV, Hall IM. Susceptibility of pupae of cabbage looper, *Trichoplusia ni* to nuclear polyhydrosis virus. I. General responses. Journal of Invertebrate Pathology. 1969;14:227–236.

Peer-review history:
The peer review history for this paper can be accessed here:
http://prh.sdiarticle3.com/review-history/26941