Pakistan J. Zool., vol. 51(2), pp 559-565, 2019. DOI: http://dx.doi.org/10.17582/journal.pjz/2019.51.2.559.565

Effect of Sub-Lethal Doses of *Beauveria bassiana* and Nitenpyram on the Development of Red Palm Weevil, *Rhynchophorus ferrugineus* (Olivier)

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Article Information
Received 06 July 2018
Revised 22 August 2018
Accepted 12 September 2018
Available online 15 February 2019

Authors' Contribution
MAS and MAQ designed the research, conducted experiments, analysed and interpreted the results and wrote the manuscripts. M Ali and M Amin assisted in designing the research. MT assisted in bioassay and provided diet to RPW. SM reviewed the manuscript.

Key words
Red palm weevil, *B. bassiana*, Nitenpyram, Development.

ABSTRACT

Red palm weevil *Rhynchophorus ferrugineus* (RPW) is a very serious pest of oil palms, coconut palms and date palms in many parts of the world. The present study was carried out to study RPW biology and development under laboratory conditions and survival against sub-lethal doses of *B. bassiana* and biorational insecticide (Nitenpyram). Sugarcane sets as alternate to date palm stem were successfully used as diet for rearing RPW. Combination of entomopathogenic fungus (Bb) and Nitenpyram (Nit) were found more lethal to survival of RPW. A reduction in pupation and adult emergence was recorded in the combined treatments. Moreover, decrease in weight gain, frass production and cumulative gain in size was found when larvae were treated with integrated effect of Bb and Nit. Depending on the lethal of treatment, development duration of RPW was disturbed. Integration of Bb and Nit delays the development and diet uptake in RPW which can be used for agent of control for this cryptic insect.

INTRODUCTION

The *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae), is a voracious feeder of palm trees (Al-Dosary *et al*., 2016; Faleiro *et al*., 2016) spread across the world. It was first reported from India in 1891 on coconut palm, *Cocos nucifera* (Lefroy, 1906), and then found on date palm, *Phoenix dactylifera* (Lal, 1917; Buxton, 1918). The *R. ferrugienus* has been reported to invade 29 palm tree species especially date palms in South East Asia, Africa, and Middle East (Dembilio *et al*., 2015; Wakil *et al*., 2015; Faleiro *et al*., 2016). This pest has been found in 50% of date-growing and 15% of coconut-producing countries in the world (Yasin *et al*., 2017).

R. ferrugienus female lay their eggs in the apertures with their rostrum in leaf base (Murphy and Briscoe, 1999). Oblong shape eggs are creamy and have glossy surface with a size ranging from 2-3 mm long (Menon and Pandalai, 1960). Eggs hatching takes three to several days depending on temperature (Murphy and Briscoe, 1999). Larvae enter the palms and make galleries by feeding inside the trunk on soft portion, tree bole and preferred upper portion of the palm. RPW attack not only the rotting part of the palms as well as feed on healthy palm trees. Owing to concealed habitat of this pest, it is very difficult to detect the attack of this pest inside the tree. Fully mature larvae spin cocoons inside leaf base and pupate (Giblin-Davis *et al*., 1996). Pupal development lasts 11-45 days (ASDPD, 2012; Faleiro *et al*., 2012). After emergence, adults live and feed inside host to complete its life cycle (Dembilio *et al*., 2011). In case of severe attack, thick yellowish brown liquid ooze out inside the tree, frass production and fermented odor is produced in attacked portion (Kaakeh *et al*., 2001) which sometimes leads to the death of tress (Abraham *et al*., 1998).

Laboratory rearing of insects is carried out with the aim to maintain species purity, selection of insect on age and sex basis and to provide successive culture for lab and field studies for pest management. Rearing of *R. ferrugineus* under laboratory conditions is laborious and expensive. Therefore, an alternate diet from locally available sources is needed. *R. ferrugineus* rearing on sugarcane stem and banana or/and synthetic diets were successfully done by several researchers in Egypt (Salama and Abdul-Razek,

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0030-9923/2019/0002-0559 $ 9.00/0

2002) while many other (Wattanapongsiri, 1966; Rahalkar *et al*., 1972, 1978; El-Ezaby, 1997; Abd El-Fattah *et al*., 2009) have reported rearing RPW on sugarcane sets, sugarcane bagasse, yeast, fresh coconut, sugarcane, methyl parahydroxyl benzoate KOH, and sorbic acid. For the rearing of *R. ferrugienus*, artificial diet was developed by El-Sebay *et al*. (2003), such as vitamins B and D, carrot, potato, agar, casein and cereals.

While going through scientific literature, great difference is found regarding the biology of *R. ferrugienus*. Alterations in life history in response to change in environmental factors are obvious in many insect populations (Whitman and Agrawal, 2009). It is the plasticity in life-history traits that makes it difficult to predict how exposure to low levels of a stressor, will influence the evolution of a population. So, the involvement of exposure to sub-lethal level of microbial entomopathogens can be of great consideration for understanding evolutionary linkages. The present research was conducted to observe the changes in the development of *R. ferrugienus* in response to sub-lethal doses of *B. bassiana* and Nitenpyram when applied alone, and combinations.

MATERIALS AND METHODS

Collection and maintenance of R. ferrugineus

A survey was conducted for collection of *R. ferrugineus* from different date growing areas of Punjab Pakistan (Multan and Bahawalpur). The various life stages of *R. ferrugineus* larvae, pupae and adult (male ♂ and female ♀) were collected from fallen and infested date palm trees. All the developmental stages were kept separately in plastic boxes and brought to Insect Rearing Lab, MNS University of Agriculture, Multan. The laboratory colony of *R. ferrugineus* was established by rearing in plastic cages (30×60×60 cm) and shredded sugar cane pieces were provided to adults for feeding, egg laying and hatching. A batch of five pairs of *R. ferrugineus* were set to mate and oviposit on sugar cane pieces (24 cm) and collected eggs after every 2 days. The deposited eggs were shifted with the help of fine brush to Petri dishes with piece of sugarcane for hatching purpose.

After 2 to 6 days (depending on temperature), newly hatched larvae of *R. ferrugienus* were transferred to rearing cage with the help of fine hair brush. Clean, fresh and infestation free sugarcane sets were offered as diet to the growing larvae of *R. ferrugineus.* From the first larval instar to adult emergence, the *R. ferrugineus* were reared individually. Small pieces of sugarcane sets (6 cm) were provided to neonate larvae to molting. Observations were made on daily basis and sugarcane pieces changed after every two days. The larvae were put into the hole made manually to facilitate the boring. For the later stage larvae (2^{nd} to 7^{th} instar), sugarcane pieces of 24 cm were provided. Larvae were transferred to the new sugarcane stems pieces after every week until pupation. *R. ferrugienus* larvae started cocoon formation from sugarcane fibers for pupation. Cocoons were collected and placed in plastic jars, sprayed with water to avoid drying. Cocoons were observed daily after two weeks for adult emergence. Emerging adults were collected and maintained at 25±2 °C, 65±5% R.H. and 12:12 D: L photoperiod in the rearing chamber (Kaakeh, 2006).

Effects of B. bassiana *and Nitenpyram on* R. ferrugineus *development*

The effects of sub-lethal concentrations of *B. bassiana* and Nitenpyram on development of *R. ferrugineus* was assessed. Second instar larvae were exposed to sub-lethal concentration of *B. bassiana* (1×10^4 conidia/ml and 1×10^5 conidia/ml) and Nitenpyram (150 µl l^{-1} and 250 µl l^{-1}). The sub-lethal concentrations were determined through preliminary experimentation. Treated sugarcane pieces were provided to larvae for feeding for 48 h and then shifted on normal untreated sugarcane sets. Dry coir was provided to each larva before pupation for cocoon formation. For newly emerging adults, shredded sugarcane pieces were offered. Three replicates of 12 insects were used for each treatment and same count of larvae fed on normal diet served as untreated check. The entire experiment was repeated twice.

Diet consumption and frass production

An additional assessments were made on the impact of sub-lethal concentrations on the diet consumption and frass production on last (seventh) instar *R. ferrugienus*; this experiment only involved single concentration of *B. bassiana* (1×10^4 conidia ml^{-1}) and Nitenpyram (150 µl l^{-1}). Seventh instar larvae of *R. ferrugienus* were exposed to single and combined sub-lethal doses of both agents (Bb+Nit) with a batch of untreated larvae as check. Before exposure, larvae were pre-weighed on electric balance and provided with normal diet. Larvae continued to feed until transferred into pupal stage and weighed just before pupation. Unused diet was collected, oven dried and weighed. From the record of oven dried diet (at 80°C) before and after feeding, the actual amount of diet consumed was calculated. Diet consumption of each larva was thus determined by subtracting the mass after feeding from before feeding estimate.

Frasss production was recorded for the diet seventh instar larvae were offered. Frass produce was separated in individual vials using a camel hair brush and weighed. The cumulative weight gain of larvae was also determined from pre-feeding and after feeding weight. Three replicates of 12 insects were used for each treatment and same count of larvae fed on normal diet served as untreated check.

Statistical analysis

Data recorded for different biological parameters (life duration for larval, pupal and adult stages, diet consumption, frass production and weights gain of *R. ferrugineus*) were subjected to analysis of variance in Minitab software (Minitab, 2007). Means were separated for significance using Tukey's HSD test at α=5 (Sokal and Rohlf, 1995).

RESULTS

Development stages of R. ferrugineus

The experiment showed that given diet of sugarcane provide sufficient nutrition to support all developmental stages of *R. ferrugienus* (Table I). However, seven larval instars were observed, and also recorded that male of *R. ferrugienus* were smaller in size and morphologically different from female due to the presence of hairs on male rostrum (snout) while female rostrum was free from hairs. Growth and development of *R. ferrugienus* (larval stage to adult) was adversely affected by application of sub-lethal doses of *B. bassiana* and Nitenpyram, significant variations were recorded for larval duration, larval weight, pupal duration, pupal weight, adult longevity and adult weight (larval duration: $F_{8,35}$ =542, P< 0.01; larval weight $F_{8,35}$ =30.1, P< 0.01; pupal duration: $F_{8,35}$ =139, P< 0.01; pupal weight $F_{8,35}$ =12.0, P< 0.01; adult longevity (female $F_{8,35}$ =150, P< 0.01 and male $F_{8,35}$ =207, P< 0.01); adult weight (female $F_{8,35}$ =11.3, P< 0.01 and male $F_{8,35}$ =26.6, P< 0.01). Increase in larval, pupal duration while reduction in adult life span was recorded for male and female when exposed to sub-lethal doses of both agents (Table II). Life duration was based on the lethal action of agent and highest effect on growth was observed in combine application of *B. bassiana* and Nitenpyram [QA (h) and NIT (l)], as compared to sole application of either agent.

Table I.- Average life stages and biological aspects of *R. ferruginueus* reared under laboratory conditions.

Stage of insect	Mean duration (days)	Size (cm)
First instar	8.10±0.36 e	0.24±0.01 j
Second instar	9.91±0.31 de	1.05±0.02 i
Third instar	10.84±0.34 cde	1.41±0.01 h
Fourth instar	12.57±0.58 cd	2.12±0.02 g
Fifth instar	13.76±0.64 c	3.05±0.02 f
Sixth instar	14.03±0.74 c	4.12±0.01 c
Seventh instar	12.91±0.62 cd	4.96±0.01 b
Pupae	21.39±0.81 b	7.79±0.01 a
Adult male	47.36±1.12 a	3.24±0.01 e
Adult female	49.28±0.82 a	3.47±0.04 d

Table II.- Effect of *B. bassiana* (1×10^4 & 1×10^5spore/ml) and Nitenpyram (150 µl l^{-1} & 250 µl l^{-}) on the development of *R. ferrugineus*. Mean sharing the same letters within each column are not significantly different at 5% level.

Treatment	Larval duration (days)	Larval weight (mg)	Pupal duration (days)	Pupal weight (mg)	Adult longevity (days)		Adult weight (mg)	
					Male	Female	Male	Female
QA (l)	84.85±0.45^{f}	3.72±0.09^{bcd}	18.55±0.12^{g}	4.22±0.03^{bc}	40.00±0.40^{de}	42.15±0.05^{cd}	1.32±0.04^{bc}	1.07±0.05^{bcd}
QA (h)	95.10±1.38^{cd}	4.21±0.12^{ab}	19.75±0.09^{f}	4.49±0.17^{ab}	38.70±0.26^{ef}	40.10±0.28^{e}	1.37±0.13^{ab}	1.28±0.02^{b}
NIT (l)	89.80±1.04^{e}	4.30±0.10^{ab}	19.90±0.12^{f}	3.90±0.08^{cde}	41.30±0.25^{cd}	42.85±0.20^{c}	1.11±0.05b^{cd}	1.05±0.03^{cd}
NIT(h)	92.40±0.98^{de}	3.84±0.07^{bc}	20.85±0.12^{e}	4.02±0.11^{bcd}	42.80±0.08^{b}	44.75±0.28^{b}	1.25±0.05^{bc}	0.94±0.03^{d}
QA(h)+NIT(l)	103.15±0.50^{b}	3.47±0.12^{cd}	22.05±0.09^{d}	3.56±0.12^{def}	41.75±0.15^{bc}	43.25±0.05^{c}	1.11±0.04^{bcd}	1.00±0.07^{d}
QA(l)+NIT(h)	105.05±0.42^{ab}	3.59±0.14^{cd}	24.20±0.1^{c}	3.42±0.06^{efg}	36.75±0.45^{g}	38.55±0.35^{f}	1.02±0.08^{cd}	0.92±0.03^{d}
QA(l)+NIT(l)	108.30±0.26^{a}	3.25±0.11^{d}	27.30±0.05^{a}	3.04±0.02^{g}	33.60±0.18^{h}	35.70±0.19^{g}	0.84±0.03^{d}	0.70±0.04^{e}
QA(h)+NIT(h)	97.30±0.36^{c}	3.54±0.07^{cd}	25.65±0.17^{b}	3.24±0.10^{fg}	38.45±0.17^{f}	41.25±0.22^{de}	1.35±0.09^{abc}	1.23±0.04^{bc}
Control	80.75±0.85^{g}	4.50±0.18^{a}	20.80±0.14^{e}	4.73±0.12^{a}	45.35±0.35^{a}	47.70±0.33^{a}	1.67±0.03^{a}	1.50±0.05^{a}
$F_{8,35}$	542	30.1	139	12.0	150	207	11.3	26.6
P	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

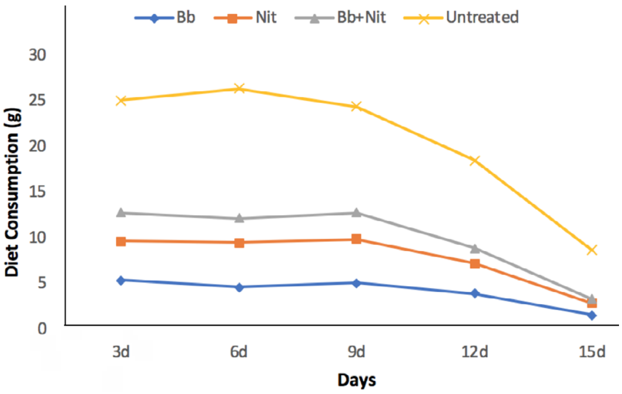

Fig. 1. Diet consumption (g) in last instar larvae of *R. ferrugineus* when treated with *B. bassiana* (1×10^4 spore ml−1) and Nitenpyram (150 µl l^{-1}) sole and combined application. Bb, *Beauveria bassiana*; Nit, Nitenpyram.

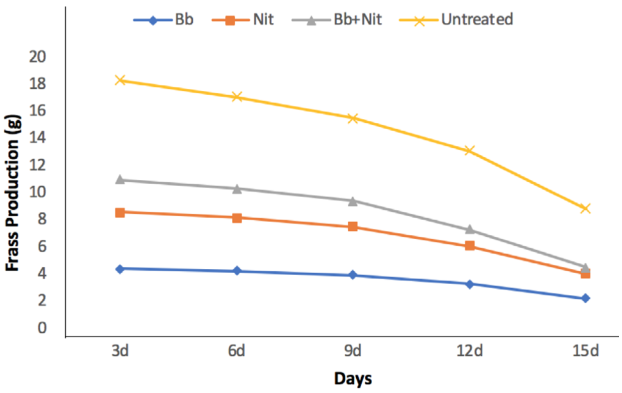

Fig. 2. Frass production (g) in last instar larvae of *R. ferrugineus* when treated with *B. bassiana* (1×10^4 spore ml−1) and Nitenpyram (150 µl l^{-1}) sole and combined application. Bb, *Beauveria bassiana*; Nit, Nitenpyram.

Diet consumption, frass production and weight gain

Following the pattern observed in growth and development, the more virulent the treatment, the more detrimental the effects observed. Based on repeated measures, diet consumption by 7th instar was significantly influenced by the treatments applied, diet consumption was lowest in integration of *B. bassian*a and Nitenpyram compared to their sole applications (Fig. 1). For the control, maximum diet was consumed than all treatments. Higher the toxicity of applied treatment, lower was food consumption and vice versa. Similarly, frass production was influenced by treatments applied. Lowest quantity of frass was produced when treated with Bb+Nit and highest in untreated check (Fig. 2). Larvae provided with diet treated with singly either with *B. bassiana* or Nitenpyram gained more weight as compared to their combined application of both (Fig. 3).

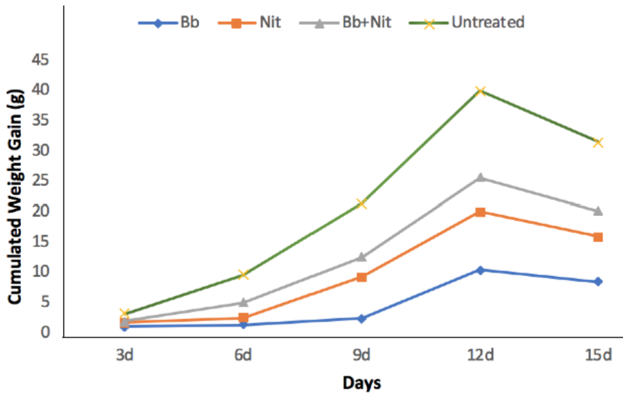

Fig. 3. Cumulative weight gain (g) in last instar larvae of *R. ferrugineus* when treated with *B. bassiana* (1×10^4 spore ml−1) and Nitenpyram (150 µl l^{-1}) sole and combined application. Bb, *Beauveria bassiana*; Nit, Nitenpyram.

DISCUSSION

Owing to larvicidal activity and less toxicity to nontarget species, EPF proved to be effective biocontrol agent for the control of insect pests (Freed et al., 2012). This is the first study to investigate the sub-lethal effect of *B. bassiana* and Nitenpyram against *R. ferrugienus*. The present study shows that sub-lethal doses of *B. bassiana* and Nitenpyram had a significant effect on the growth and development of target insect. By increasing selection pressure through physiological process, pathway is set for resistance development. Present study supports the hypothesis that a single target-site mutation confers a high level of resistance. Retarded growth in insects in response to physiological stressor is of key consideration and should be carefully monitored while finalizing doses for bioassays or designing future studies.

Low doses of insect stressors like insecticides and microbial entomopathogens are considered as a tool to decrease the selection pressure and hence to delay the onset of resistance (Helps *et al*., 2017). In the light of present investigations, our results revealed that lethal effect of *B. bassiana* and Nitenpyram caused reduction in life span, food consumption, weight gain of the insect. Different biotic and abiotic factors like diet and temperatures affected growth and survival of the insects. Salama *et al*. (2009) observed that larval stages may last for 24-128 days, whereas number of larval instar depend upon temperature and diet quality.

For the successful completion of insect life cycles and reproduction growth and development are very important phenomenon. Insect growth and development may stop if any of factors like malnutrition, natural enemies or environmental stresses are involved. In this regard insect larval stages are susceptible towards such phenomena (Marzban *et al*., 2009). Many scientists working on *R. ferrugienus* rearing and highlights the different natural and artificial diets for its successful rearing. But it is hardly to rear on artificial diet due to difficulty in preparation and change diet for insect on daily basis. However, various diets are contaminated with microorganisms and whole colony of insects are badly infected and destroyed (Aldawood and Rasool, 2011). The appropriate diet is required for the successful rearing and good health of the insects. Sugarcane is the ideal diet for the rearing of *R. ferrugienus* and has the advantages that it is less expensive, no labor cost, and free from microorganism contamination. Most of researcher easily rear the RPW on sugarcane and observed several generations and various larval instars.

Low doses of insect stressors impede development, retard the growth and survival but exceptionally enhance reproductive performance. Such effects are often species-specific, depending on the developmental stage, sex and target insect. However, more concerned phenomenon is the alternation in immunity pathways and changes in behaviour. During chemical communication, these changes can act as info-disruptors, blocking signal transmission and perception during chemical communication. Often the low concentrations can readily lead to selection of resistance in pest insects (Casida and Durkin, 2013) and the changes in insects' physiology, behaviour and communication (Navarro-Roldán and Gemeno, 2017; Tappert *et al*., 2017).

In the present study, *R. ferrugineus* was successfully reared on sugarcane stems for one generation without any apparent deformity or difficulty in rearing under laboratory conditions which is one of the natural food for this pest. In laboratory conditions, in contrast to natural palms, sugarcane is one of the best natural diet for the successful rearing of all stages of *R. ferrugienus* especially for pupation (Kakeeh *et al*., 2001; Resh and Carde, 2009; Mahmoud *et al*., 2015). Our result shows larvae of *R. ferrugienus* were successfully reared on sugarcane sets and using the fibers contents of sugarcane, cocoon formation for pupation was a notable success. Jaya *et al*. (2000) reared *R. ferrugienus* on sugarcane stem and observed 7 instars while Martín and Cabello (2006) contrarily noted 17 instars. Nirula (1956) surprisingly mentioned three larval instars in RPW when reared on meridic diet, however, Dembilio and Jacas (2011) described 13 instars from in RPW reared in *P. canariensis* trees. *R. ferrugineus* takes 4 months to complete its life cycle and larvae may molt 7 times. Similar result was obtained by Ajlan (2008) who reported that *R. ferrugienus* complete its life span within 4 months, while Wattanapongsiri (1966) reported that *R. ferrugineus* larvae takes 25 to 105 days during its larval stage and 9 to 20 times molting recorded during its larval duration. Life cycle of *R. ferrugineus* and number of larval instar depends upon temperature, food type/diet material and other life parameters was also affect its behavior (Stamp, 1990). In case of other insects, Dyar's law is very helpful to determine the number of instars by measure the width of head capsule (Klingenberg and Zimmermann, 1992). There have been successful efforts to rear many agriculture important insect pests on artificial diet and obtained several successive generations, but in some cases, loss of stability and reproduction caused longer development times and reduction in fecundity (Coudron *et al*., 2002).

CONCLUSION

Our results concluded that sugarcane is the best food medium between the natural diet for the successful rearing of *R. ferrugienus* under laboratory conditions in Pakistan. The sub-lethal doses of *B. bassiana* and Nitenpyram also effect the reduction in life span, food consumption, weight gain of the insect. Therefore, *B. bassiana* and Nitenpyram fraction could be reliable management strategy that can highly recommend for the control of *R. ferrugienus*.

ACKNOWLEDGEMENT

This research was funded by the grant of Higher Education Commission (HEC), Islamabad, Pakistan under the project (SRGP No. 1181)

Statement of conflict of interest

The authors declare no conflict of interest.

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