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Evaluation of Entomopathogenic nematode and fungi alone and their combination against red palm weevil, *Rhynchophorus ferrugineus* (Olivier)

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Abstract
The Red Palm Weevil *Rhynchophorus ferrugineus* is a globally obnoxious insect pest of date palm (*Phoenix dactylifera* L.). Different concentrations of *Beauveria bassiana* and single concentration of *Heterorhabditis bacteriophora* and their combination was applied on 2nd, 4th and 6th grub instars. The data of mycosis, sporulation, number of nematode produced, number of grubs infected and grub mortality was observed after 7, 14 and 21 days of treatment and data analyzed by probit analysis. In combination (Bb2+Hb) 100% and 95.03% mortality of 4th and 6th instar after 21 days. Maximum sporulation was recorded 190.00% in 2nd instar grubs when Bb1 applied alone, while least sporulation was 128.05% observed in adult stage when Bb2+Hb was used in 6th instar. Maximum larvae affected by nematodes are 75.92% in 2nd instar grubs when Hb applied alone, while least was number of Larva 32.61% recorded in adult stage when Bb2+Hb was used in 6th instar.

Keywords: entomopathogenic fungi, entomopathogenic nematode

1. Introduction
Rhynchophorus ferrugineus (Coleoptera: Curculionidae) locally known as Red Palm Weevil (RPW) is a notorious and damaging insect vermin of date palm. This specie is polyphagous and is found in many countries including Pakistan [8]. Red palm weevil also recognized as Asiatic palm weevil, Date palm weevil or Indian palm weevil [17]. In existing situations it is extensively scattered in Oceania, Europe, Africa and Asia [15]. *R. ferrugineus* was first reported in the UAE in 1985 [12]. Later in Pakistan in 1917 it was described as arduous and dangerous pest of palm cultivations [28]. The countries producing more than 50% of dates [14] that account 68% of world date's production [13] have observed the prevalence of RPW. The damage is completely done by the grubs which feed on the tissues in the stem and in the center of the tree.

The grub feed internally by boring the tissues of stem and eventually kills the palm tree. In case of heavy infestation all the life stages (eggs, grubs, pupa and adult) of weevil remain inside the palm trunk. During the whole life span a single female can lay 58-760 eggs [17, 18]. Damaged or injured palms are good breeding sites for egg laying by female, hatched grubs make holes into the fresh tissues and enter to the bud region and centre of the crown where they feed for two to four months and finally kill the host plant [31]. Red Palm Weevil reported to damage 17 species of palms worldwide. Clear symptom of attack in date palm may be exuding out of fluid from the trunk or stem. Young palm trees between the age of 5 and 20 years old are mostly attacked by the *R. ferrugineus* and infestation is directly related with palm tree density [16].

Symptoms that are produced by the *R. ferrugineus* includes tunnel formation on the stem and leave petiole base, thick brownish liquid exude out from the holes made by weevil, fermented odor, dried offshoots appears, sound produced by the grubs, empty pupal coverings fall down and breaking of the stem and infested parts occur [2]. The present management approach involves an IPM such as insecticide application, mass trapping, monitoring and early detection [18, 29]. Chemical insecticides are very effective for the elimination of RPW but these insecticides are exist for short period so they need to be applied occasionally as they have negative effects on human health and resistance appear in the insect [26, 18, 20]. Insecticides are applied as curative and preventive treatments to reduce the spread of weevils [1].

Due to the concealed behavior of this pest, insecticides are used repeatedly for the time period for efficient control of recognized RPW's populations [29]. The progress of a biological control for a successful IPM requires recognition of the natural enemies of the RPW [29]. Biological control agents such as microorganisms, microbial products and organic fertilizers have been used recently as alternatives to chemical control [33].

A superlative and alternative to the chemical control of Red palm weevil is the application of EPNs (entomopathogenic nematodes) [26, 11]. EPNs are host specified, target oriented and environmentally safe and are mass produced in liquid suspension and in recent times their costs of production have been considerably decreased [9, 10]. The third juvenile stage also called infective stage or dauer juvenile (DJ) live externally on insect body. From any orifice (spiracles, anus and mouth) DJs enter in the host and develop into the parasitic/infective stage. Within the gut of the nematode gram-negative bacteria are present and cause the death of the insect host [22]. *Beauveria bassiana* (Clavicipitaceae) an important mycopathogen; entomopathogenic fungi, is saprophytic in nature and grow on insect cadavers, it builds conidial spores on the surface of host cuticle and rapid germination of new conidial spores in the body of host occur through formation of yeast like cells known as blastospores [38]. An entomopathogenic fungus actually just needs to have contact with the insect body, after which it penetrates through cuticle. Host of the fungi can be diseased by direct dealing or can also be transmit from infested insects to untreated and infection transferred by more production of spores [30].

Entomopathogenic fungi caused 50-100% grub mortality of those adults which are contaminated with *B. Bassiana* and *M. anisopliae* [23]. The focusing objective of this research was to explore the potency of *Heterorhabditis bacteriophora* and *Beauveria bassiana* sole or in combination against the *Rhynchophorus ferrugineus.*

2. Materials and Methods

2.1 Rearing of red palm weevil

A survey was conducted to collect Red palm weevil's population from different districts of Punjab including Jhang, Bhakkar, Layyah, Muzafargarh, Multan, Lodhran and Bahawalpur, Pakistan. From this survey, all stages, e.g. grubs, pupae and adults was collected from date palm orchards of respective districts and population was brought to Microbial Control Laboratory, University of Agriculture, Faisalabad. For rearing purposes, insects were kept in incubator provided with fresh sugarcane stems for feeding of the grubs and for pupation and shredded sugarcane pieces were offered as diet for adults. The rearing conditions were maintained at 27±2ºC and 65±5% R.H. and 12:12 D: L photoperiod.

2.2 *Beauveria bassiana* culture and Nematode culture

The entomopathogenic fungi *B. bassiana* and Nematode culture was obtained from culture collection of Microbial Control Laboratory, Department of Entomology, university of Agriculture Faisalabad, Pakistan.

2.3 Bioassay of *Beauveria bassiana* (alone) against 2nd, 4th and 6th instar grubs

2nd, 4th and 6th instars of red palm weevil were directly immersed in groups of 10 for 60s in a conidial aqueous suspension of 1×10^6 and 1×10^7 conidia/ml or in a control aqueous solution. Both treated and control solutions contained 0.01% Tween 80. After treatment, grubs were individually transferred onto a moistened filter paper in a Petri dish (2.5 cm diam.) for 24 h within the cane for feeding purpose. Mortality was recorded after 7, 14 and 21 days.

2.4 Bioassay of *H. bacteriophora* (alone) against 2nd, 4th and 6th instar grubs

Experiment was conducted in petri dishes (9 x 1.5 cm) lined with moistened filter paper. Each dish was inoculated with 100 IJs in 1 ml of water and was given 30 minutes to distribute on the filter paper. Five grubs of both instars were released in petri dishes. Each dish was provided with a piece of sugarcane as a grub food. Grubs were feed on untreated sugar cane shreds, used as control. Mortality was checked after 7, 14 and 21 days.

2.5 Bioassay of *B. bassiana* and *H. bacteriophora* (combined)

Combined efficacy of *H. bacteriophora* and *B. bassiana* against 2nd, 4th and 6th instar grubs of red palm weevil was evaluated. Sole and Combinations were made as Bb1, Bb2, Hb, Bb1+Hb and Bb2+Hb. For combined treatment firstly, group of 10 grubs of 2nd, 4th and 6th instars were directly dipped in *B. bassiana* aqueous conidial suspension of 1×10^6 and 1×10^7 conidia/ml for 60s. Then this fungus treated grubs were air dried for 10 minutes and simultaneously *H. bacteriophora* inoculated with 100 IJs in 1 ml of water on petri plates lined with filter paper and *B. bassiana* treated grubs released into these petri plates. Both control and treated grubs were dipped in 0.02% Tween 80. Data regarding grubs mortality observed after 7, 14 and 21days.

2.6 Mycosis and Sporulation

Dead grubs or cadavers were collected on daily basis and were surface sterilized with 0.05% sodium hypochlorite followed by sterilized distilled water three times, placing them on SDAY plates. Cadavers were incubated at 26±2ºC, 75% RH for 10 days. The data for Mycosis was estimated by observing cadavers external mycelia growth using a stereo-microscope. The data for Sporulation was estimated by mixing mycosed cadavers in a beaker containing 25 ml sterile distilled water and a drop of Tween 80 After stirring the solution with a magnetic stirrer for 5 min, conidia were counted by using a heamocytometer under the compound microscope).

2.7 Nematode production

H. bacteriophora suspensions were prepared with a concentration of 100 IJs in glass jars and 1 ml of suspension was poured into the cylindrical plastic cups lined with damp Whatman filter paper. The top of the cups were covered with a fine mesh in order to avoid the insects to escape. After pouring nematodes 30 minutes time was given for evenly distribution on filter paper. A small piece of artificial diet 2×2 cm^2 was placed in middle of the cups as a food source for grubs and provided with new food after every day. In each cup one 4th instar grubs of *R. ferrugineus* from each population was placed on the top of the filter paper. Same procedure was repeated for adult population and shredded sugarcane pieces were offered as a food source instead of artificial diet. Each treatment was replicated three times, while control treatment received 1 ml of water. The cups were placed in incubator at 25±2 °C and 70±5% RH at 12: 12 (D: L) hours photoperiod. Mortality data was recorded after 7, 14 and 21 days after treatment and whole bioassay was repeated thrice to avoid the pseudo replication. Dead individuals were

transferred to the modified White traps and left for 10 more days for IJs emergence. The insects exhibiting typical odor and colour (signs for nematode infestation) were considered to be killed by nematodes.

Concentration of IJs was measured by 1 ml sample from the final solution and counting IJs with the help of a Peters' slide and microscope.

2.8 Statistical analysis

The mortalities mean will be corrected by using Abbott's (1925) formula. At day interval means will be compared by using Tukey's test at significance level of α= 0.05%.

3. Results

3.1 Mortality of 2nd, 4th and 6th instar grubs of *Rhynchophorus ferrugineus*

The grub mortality increased with the increasing time interval in all treatments. The time interval significantly affected the grub mortality and significant time × treatment interaction was found. The alone application of *H. bacteriophora* resulted in increasing mortality of *R. ferrugenious* by increase in day intervals, followed by *B. bassiana*. Whereas, in the combination (Bb2+Hb) 100% mortality was seen after 21 days of 2nd instar grubs (Table- 1). In overall experiment the mortality rate of 2nd instar grub did not differ significantly in all combinations. The higher mortalities were found with the increase in time interval in both sole and combine applications *H. bacteriophora* and *B. bassiana*. In the combination (Bb2+Hb) 100% mortality was seen after 21 days of 4th instar grubs (Table- 1). Mortality rate was gradually increasing after day interval.

3.2 Mycosis and Sporulation in 2nd, 4th and 6th grubs of *R. ferrugineus*

Maximum mycosis was recorded 78.33% in second instar grubs of *R. ferrugineus* when Bb1 applied alone, while least mycosis was 30.86% observed in 6th instar when Bb2+Hb nematode interaction was used (Table- 2). Maximum sporulation was recorded 190.00% in second instar grubs of *R. ferrugineus* when Bb1 applied alone, while least sporulation was 128.05% observed in adult stage when Bb2+Hb nematode interaction was used in 6th instar (Table 2).

Table 1: Mean (±SE) Mortality of 2nd, 4th and 6th instar grubs of *Rhynchophorus ferrugineus* due to bio efficacy of *Heterorhabditis bacteriophora* and *Beauveria bassiana*.

	After 7 Days of Treatment			After 14 Days of Treatment			After 21 Days of Treatment		
EP	**2nd instar**	**4th instar**	**6th instar**	**2nd instar**	**4th instar**	**6th instar**	**2nd instar**	**4th instar**	**6th instar**
Bb1	15.01±2.19b	13.51±2.93b	11.45±2.38b	37.00±2.94c	31.48±2.37c	28.74±2.51c	25.13±3.76c	20.56±2.43b	18.08±2.42c
Bb2	25.13±3.76b	20.12±2.16b	18.82±1.63b	49.34±3.26c	40.21±3.0bc	36.42±2.69bc	33.31±2.02bc	30.69±2.62b	24.34±3.65bc
Hb	19.53±2.09b	16.98±2.63b	14.19±2.11b	42.57±2.40c	34.98±3.24c	32.85±2.96c	31.63±2.38bc	26.37±2.47b	21.51±2.07bc
Bb1+Hb	34.81±3.56ab	29.63±3.00ab	21.65±2.982b	71.08±3.54b	63.73±2.45b	55.39±3.84b	46.05±3.47b	42.32±2.26ab	39.28±2.89ab
Bb2+Hb	51.60±2.41a	44.47±3.10a	38.36±3.17a	100.00±0.00a	100.00±0.0a	95.03±3.97a	65.69±3.69a	58.06±3.01a	51.91±3.75a
F	6.95	7.60	5.16	21.17	19.32	23.25	10.3	6.80	7.98
P	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

EP mean Entomopathogens,

Table 2: Mean (±SE) Mycosis and Sporulation of *Beaveria bassiana* at 2nd, 4th and 6th instar grubs of *Rhynchophorus ferrugineus*.

	Mean (±SE) Mycosis of *Beaveria bassiana*			Mean (±SE) Sporulation of *Beaveria bassiana*		
Entomopathogen	**2nd instar**	**4th instar**	**6th instar**	**2nd instar**	**4th instar**	**6th instar**
Bb1	78.33±2.38a	75.92±1.76a	68.51±1.60a	190.00±2.70a	180.27±2.12a	175.89±2.54a
Bb2	70.54±3.15ab	67.31±2.53ab	63.96±2.91ab	170.44±2.50b	158.12±1.00b	149.54±1.09b
Bb1+Hb	56.11±0.06ab	45.70±2.94ab	42.19±2.87ab	165.67±1.41b	151.33±2.21b	144.62±2.12b
Bb2+Hb	41.52±1.61b	33.82±1.86b	30.86±2.39b	147.30±2.51c	132.72±1.98c	128.05±1.14c
F	4.01	3.25	3.37	27.3	29.6	31.9
P	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

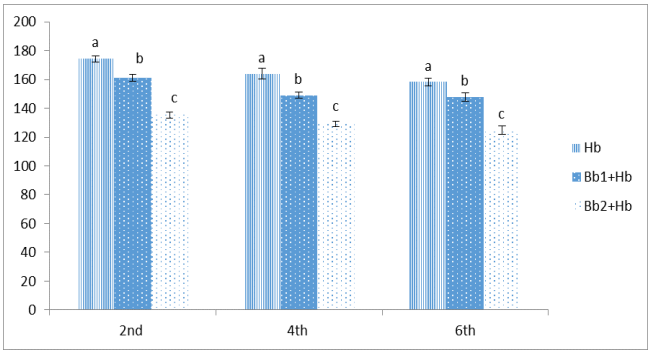

Fig 1: Mean (±SE) Number of nematode produced at 2nd, 4th and 6th instar grubs of *Rhynchophorus ferrugineus*

Maximum Number of nematode produced by nematodes was 174.41% in second instar grubs of *R. ferrugineus* when Hb applied alone, while least Number of nematode produced by nematodes 124.68% observed in adult stage when Bb2+Hb nematode interaction was used in 6th instar (Fig-1). In case of larvae effected by nematodes the maximum number of Larvae affected by nematodes are 75.92% in second instar grubs of *R. ferrugineus* when Hb applied alone, while least was number of Larva 32.61% recorded in adult stage when Bb2+Hb nematode interaction was used in 6th instar (Fig-2).

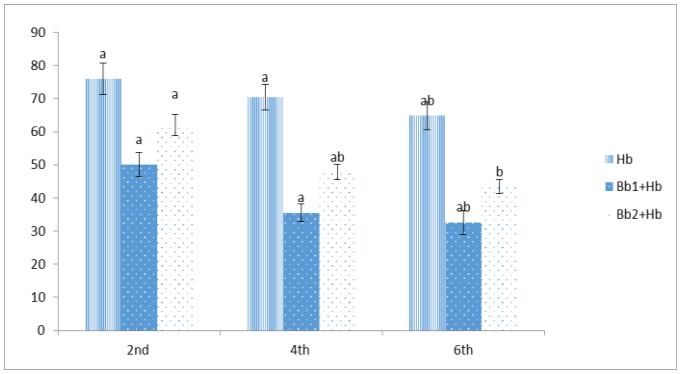

Fig 2: Mean (±SE) Grubs affected by nematodes at 2nd, 4th and 6th instar grubs of *Rhynchophorus ferrugineus*.

4. Discussion

Biological control measures to annihilate the *Rhynchophorus ferrugineus* (RPW) in date palms are limited due to the cryptic behavior of this pest and also inadequate data about biology, ecology and behavior of Red Palm Weevil. For effective biological control, selecting the veracious species against a specific insect pest in a certain environment is much important [32]. Fungal spore easily penetrate into insect cuticle and upon the successful penetration the fungi produced extensively vegetative growth in haemolymph which deplete the nutrition and ultimate death of host occur, hypothetical proposed by many scientist [19, 6, 4].

In this experiment also the alone and combined bio efficacy of *Heterorhabditis bacteriophora* and *B. bassiana* against red palm weevil was determined. Finding revealed that both bio control agents enhanced the effects of each other. The mortality ratio obtained was much high when combined EPF + EPN were applied as compared to alone application. Similarly Synergistic effects obtained from the integration of nematodes with other entomopathogenic fungi and bacteria have been described in many studies [24, 25].

Combination of several control agents that interact with each other give positive response and increase their virulence against the target insect pests as compared to the individual effect [27]. Lethal concentrations of different microbial agent impact negatively on the diet consumption of insects that ultimately effect on the grub development. Grub mortality occur when insect ingest the different concentration of pathogen [27]. In present research illustrated that combined applications of *Heterorhabditis bacteriophora* and *Beauveria bassiana* can work synergistically against Red Palm Weevil *Rhyncophorus ferruginesis*. Our results are supported by Ansari [5] who conducted an experiment to check the interaction of EPNs and EPF against *Otiorhynchus sulcatus.*

Butt [7] explained the possible reason that higher concentrations of fungi (Ma) enhanced the possibility for the insect to contact with conidia, after attachment conidia develop and enter into the body of host. It is assumed that at higher concentrations of fungi this process accelerated has impact on bacteria related with nematodes and subsequently inhibit the nematode production. Mostly *H. bacteriophora* combined with *B. bassiana* enhance the lethal efficacy of each other [35]. This study corroborates earlier work, targeting *H. philanthus* Füessly white grubs, that showed that both the type and intensity of the interaction depends on the EPN species and the timing of its application [5]. Combined application of *H. bacteriophora* and *M. anisopliae* isolate enhanced grub mortality of *C. curtipennis*, in a synergistically or additively manner [3]. Results indicated that if EPF is applied first, it acts like stressor to host insect, thereby enhancing the bio-efficacy and potency of EPN. It was claimed that, those insect pests that are being stressed are much more susceptible to pathogenicity [34].

5. Conclusions

Management of RPW in date palms cultivation is an intricate challenge that necessitates sustainable RPW-IPM. Our results proved that combination of *Heterorhabditis bacteriophora* and *B. bassiana* were highly significant from their alone and combined concentration for 2nd, 4th and 6th instar grubs of red palm weevil and should be strongly considered in the Integrated Pest Management of *R. ferreugienus.*

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