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Comparative effectiveness of entomopathogenic nematodes against red palm weevil (*Rhynchophorus ferrugineus*) in Pakistan

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Abstract
In this investigation the effectiveness of EPNs (entomopathogenic nematode species) including *Steinernema carpocapsae*, *Heterorhabditis bacteriophora* and *Steinernema feltiae* on the *Rhynchophorus ferrugineus* larvae and adults were scrutinized. While during bioassays, plastic boxes of 9x5x5 cm size were used. Whatman filter paper was retained at the base of each culture box and 3rd, 6th and 10th instar larvae ofred palm weevil (RPW) (*R. ferrugineus*) were placed. EPNs were inoculated to weevil larvae of mentioned in stars and also to the adults at the concentration level of the 100 IJs/larva+adult and then incubated at temperature of 25°C. Later the EPNs inoculation, larval instars and adults were tested after 12 hours of time length and their mortality were noted. This investigation was terminated at the end of 8th day and consequences were assessed. All of the mentioned EPNs applied in this experiment resulted variant mortality on each larval instar and adult stage of red palm weevil. The maximum and minimum %age mortality of *R. ferrugineus* at 3rd and 10th larval instars were 96.5% and 88.17% by inoculation of *S. carpocapsae*, 85.75% and 74.4% by application of *H. bacteriophora* while 38.68% and 35.35% by employing *S. feltiae* respectively. On the other hand, minimum mortality of adult red palm weevil by application of mentioned EPNs was noticed. Furthermore, we observed increasing trend of %age mortality of *R. ferrugineus* populace after time length of 12 to 192 hours (8 days) by utilization of EPN species. At the end, we resulted that the credible application of EPNs against palm weevil should be debated and employed for monitoring this notorious pest.

Keywords: *R. ferrugineus*, Mortality, *S. carpocapsae*, *S. feltiae*, *H. bacteriophora*, EPNs

1. Introduction
The date palm (*Phoenix dactylifera*), an ancient plant was reported before 5000 years ago, heterogeneous in nature and having no propagation ability like other plants [16]. The attack of insect pests and diseases exerts severe (30%) yield losses around the world [11]. It was reviewed that *R. ferrugineus* has been spotted globally in range of 15- 50% in case of coconut producing and date-growing countries respectively [35].

The insect pests of coleopterans are placed among the rapacious pests of economically imperative crops. Among these invasive pests, *R. ferrugineus* is regarded as the extremely dangerous tissue borer, firstly detected on *Cocos nucifera* from southern parts of Asia, later on it became the pest of Middle East [14]. Afterward, infestations of the RPW were reported in India and different districts of Pakistan including Muzaffargarh, Dera Ghazi khan and Multan [26]. After 2 years, this pest was also identified in the orchards of date palms from Iraq. RPW exert massive losses ($US 130 million) in Middle East [12, 14]. It has ability to destroy 29 species of palm trees most importantly date palms, the significant crops in Africa, South East part of the Asia and Middle East [7, 13, 32]. Place of original reported home of RPW is particularly southern parts of Asia, destroy a number of coconut plantations [31].

In *P. dactylifera*, control methodologies circle around the usage of food lured pheromone traps, while in case of *P. canariensis* (Canary Island date palm), palm weevil control is mostly undertaken by the utilization of insecticides. Both, the environmental as well as non-target side effects claim for development of management practices which are sustainable and environment friendly [7].The RPW pest shows cryptic behavior so, it is the reason that synthetic pesticides were found bogus for its management [2]. Alternatively, microbial control agents' including entomopathogenic fungi (EPFs) and nematode (EPNs) shows great potential against different insect pests [19, 29, 33].

From the past 2 decades, the implementation of entomopathogenic nematodes (EPNs) has been increased to control insect pests as a biological control agent [10, 21]. Unquestionably, investigators expanded the scope of entomopathogenic nematodes for sustainable management of plant nematodes, soil inhibited pathogens of plants, mollusks and the most importantly insect pests [6]. Up till now, 30 families of EPNs are identified to have link with insect pests, particularly seven out of thirty families including Heterorhabditidae, Neotylenchidae, Steinernematidae, Allantonematidae, Rhabditidae, Mermithidae and Sphaerulariadae have great focused attention to control different pests. Among them two Steinernematidae and Heterorhabditidae shows great potential against the Red palm weevil [17, 35]. Entomopathogenic nematodes with their concomitant bacteria were found target specific and have no negative impacts on plants and mammals [3, 5]. A large number of researchers examined the efficacy of EPNs in laboratory as well as field conditions [1, 8, 22, 27].It is reviewed that EPNs from two genera including steinernema and Heterorhabditis are latent MCAs (microbial control agents) and also documented that number of targeted pest exposed to be vulnerable to EPNs has sustained to rise[20].

The objective of the current study was to evaluate the efficacy of three entomopathogenic nematode species including *S. corpocapsae, S. feltiae* and *H. bacteriophora* against the invasive palm pest *R. ferrugineus.* The experiment about the mortality of RPW was conducted in laboratory conditions in a special relation to the time interval for the efficacy evaluation of entomopathogens. The present study results will suggest the most appropriate doses and species of EPNs as well as suitable times for future management of RPW in field conditions.

2. Materials and Methods

2.1 Collection and rearing of Red palm weevil

R. ferrugineus populations were collected from the 4 provinces of Pakistan including Baluchistan, Sindh, Punjab and KPK by using pheromones (ferrugineol & ferrugineone) traps during the surveys conducted in 2015-2016. Various RPW developmental forms including larvae, pupae and adult were picked from infested trees directly while adults were collected from the pheromones traps. All the developmental forms of RPW were placed independently in plastic jars in Integrated, Genomics, Cellular, Developmental and Biotechnology (IGDCB) Laboratory at Post Graduate Agricultural Research Station (PARS), University of Agriculture, Faisalabad, Pakistan. The collected larvae were fed on the pieces of sugarcane sets. After the last instars larvae, pupation took place inside the sugarcane sets. Fresh shredded sugar canes were kept in separate plastic boxes (60x60x30 cm) and adults (males and females) were released in these boxes for the purpose of oviposition. The pupal cocoon was placed in jars for the emergence of adult weevils. The eggs of RPW were incubated by providing 25±2 ºC temperature with 65±5% relative humidity for 12D: 12L hour photoperiods. The neonate larvae were also allowed to feed on sugarcane pieces and they feed until pupation. Afterward, they were placed in adult jars for mating, feeding and oviposition purposes again. The new RPW colony was established in boxes sized (30×60×60 cm) with a mesh gauze (60 cm) covered led in the middle portion (10 cm) used for aeration purpose. After 3 days, the diet of adult was replaced with the pieces of sugarcane.

2.2 Entomopathogenic Nematode

Infective juvenile (IJs) culture of *S. carpocapsae, H. bacteriophora* and *S. feltiae* was purchased directly online from the storage unit of Sc Garden NemaSeek™, USA (10 x 10^6 nematodes for $30 each). They were assessed against the 3rd, 6th, and 10th instars of larvae as well as adults of RPW. Firstly, the entomopathogen's culture was sustained in *Galleria mellonella* L. (Lepidoptera: Pyralidae) 5th instar larvae according to the standard protocol of Kaya and Stock [18]. Infective juveniles were assembled by implementing white traps as indicated in Fig.1. They were harvested and put in storage with distilled sterilized water (10-15^{0}C) about two days before applications [34].

Fig 1: White trap collection of entomopathogenic nematodes (EPNs) from 5th instar *Galleria mellonella*

2.3 Effectiveness of EPNs on adults and larval population of RPW

For efficacy assays, Whatman filter papers were placed on the base of 9x5x5 cm sized plastic culture boxes and a 3rd, 6th and 10th instar larva of *R. ferrugineus* were put on each box. Each larvae and adult of RPW was inoculated with 100 IJs at 25°C incubation in separate petri plates. Infected larvae were checked at 12 hours duration, mortality on larvae and adults was recorded upto 8th day. Interaction between the infection and time factor was also measured by measuring the percentage mortality after 12 hours of duration. 24 *R. ferrugineus* larvae were used for each EPN species, and the bioassays were repeated thrice. To be sure that the mortality was caused by EPNs, infected larvae were put on White trap and the emergences of IJs were observed.

3. Results

Variant mortalities in case of red palm weevil larvae of 3rd, 6th and 10th instar (Fig 2) instigated by entomopathogenic nematode species (EPNs) were detected. Larvae of the control group were merely treated with water and exhibited no larval mortality. In our experiment disparate mortality percentages (Fig 3) of three *R. ferrugineus* larval instars (3rd, 6th and 10th) were spotted when inoculated by employing three EPNs strains. The %age mortality (Fig 3) of *R. ferrugineus* population at 3rd, 6th and 10thlarval instars caused by application of *S. carpocapsae* was 96.5%, 94.7% and 88.17% respectively. While the %age mortality (Fig 3) of *R. ferrugineus* population at 3rd, 6th and 10th larval instars triggered through application of *H. bacteriophora* was 85.75%, 78.15% and 74.4% respectively but in case of *S. feltiae* the percentage mortality of the weevil populace at

above mentioned three instars was 38.68%, 36.35% and 35.35% correspondingly. Moreover, minimum mortality of 3.07% was noted at adult stage of palm weevil population due to *S. carpocapsae* as shown (Fig 3) but 0.66% was due to *H. bacteriophora* and no mortality (0%) was noticed due to inoculation of *S. feltiae* strain. While those larvae that were powerless to move even by punching with a needle were thought to be dead.

A **B** **C** **D**

Fig 2: Pictures showing different instars of *R. ferrugineus* larvae that were employed for inoculation by EPN species: A= 3th instar larvae; B= 6th instar larvae; C= 10th instar larvae and D= adult *R. ferrugineus* population.

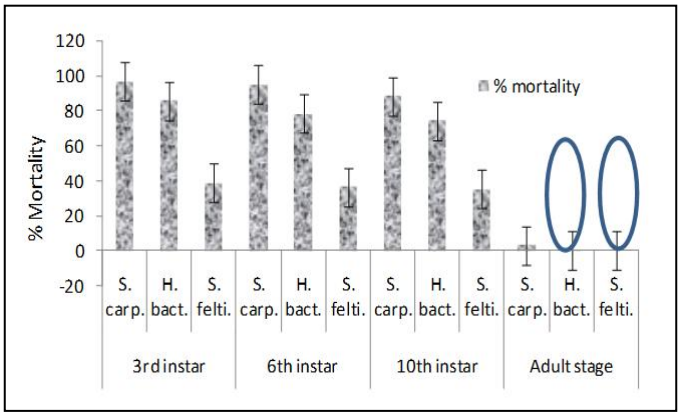

Fig 3: %age mortality of *R. ferrugineus* at different larval instars and their adult stage by inoculation of various EPNspecies (*S. carpocapsae*, *H. bacteriophora* and *S. feltiae*)

We also studied the interaction of aforementioned nematode strains (%age infestation) along with time factor against the 5th instar larvae of RPW (Fig. 4). Minimum mortality after 12 hours (time length) of EPNs application was 81%, 68.1% and 19.83% shown by *S. carpocapsae*, *H. bacteriophora* and *S. feltiae* respectively. With the passage of time we observed increasing trend of %age mortality of *R. ferrugineus* population. After the 24 and 36 hours of EPNs applications, mortality observed was 83.63%, 69.56%, 23.33% and 84.43%, 71.20% and 24.23% by *S. carpocapsae*, *H. bacteriophora* and *S. feltiae* respectively. However, after time period of 192 hours (8 days), our results indicated maximum percentage mortality of 98.9%, 86.9% and 38.53% from above mentioned strains respectively.

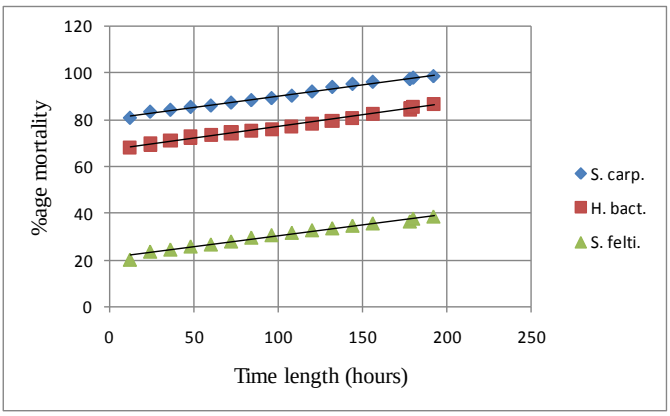

Fig 4: Picture showing the interaction of time length with %age mortality of *R. ferrugineus* against 5th larval instar by employing three EPN species

4. Discussion

Our present investigation was about *R. ferrugineus* interaction among three aforementioned EPN species. The literature provides lot of evidences in support of EPNs efficacy for causing mortality of several insect pest including red palm weevils (*R. ferrugineus*). Mahmoud [23] reviewed the application of EPNs for bio-control of economically important insect pests, stated them as potential agents of virulence and quick killer of their hosts.Our current study revealed that maximum mortality (96.5%) of *R. ferrugineus* larvae was carried out as a consequence of *S. carpocapsae* inoculation. Multiple information have been published in the last few years about the *S. carpocapsae* in order to investigate its effectiveness against this cryptic pestin Spain [8, 9] and Italy [25] which described that this nematode specie prior the discharge of its bacteria (symbiotic), weaken and elude the immune confrontations of such weevils, having the intention to position a promising environs for its bacteria accountable for the mortality of targeted insect. Our results coincide with those already discussed by Triggiani *et al.* [30] in Italy, where 100% mortality was obtained by the applications of *S. carpocapsae* against the *R. ferrugineus* larvae. The maximum mortality (91.4%) of *R. ferrugineus* larvae through utilization of *S. carpocapsae* is also reported by Gozel *et al.* [15]. Similarly, %age efficacy of *S. carpocapsae* achieved in curative as well as in preventative treatment was 80% and 98% respectively [22]. Manachini *et al.* [24] documented that mortality in case of *R. ferrugineus* shows progressive trend via dosage as well as time length to *S. carpocapsae.* Santhi *et al.* [27] stated IJs of *S. carpocapsae* as more potential agents than that of *H. bacteriophora* to reach their host of interest (*R. ferrugineus*). In present study, beside the *S. carpocapsae* the maximum percentage mortalities of red palm weevil larvae by inoculation of *H. bacteriophora* and *S. feltiae* were 85.75% and 38.68%respectively.While, %age deaths by means of *H. bacteriophora* and *S. feltiae* stated by Gozel *et al.* [15] were 93.5 and 36.4% respectively. On the other hand, *H. bacteriophora* resulted the maximum larval mortality in the range of 93-100% as already mentioned by Triggiani and Tarasco [30]. Recently in Pakistan experiments were conducted to evaluate the effect of microbial agents (including *H. bacteriophora*) for growth as well as development of *R. ferrugineus* (larvae to adult stage) resulting adverse effects. When larvae of weevils were treated via sub-lethal doses of *H. bacteriophora*, substantial variations were noted for larval duration, weight as well as adult longevity and weight [33]. Such kind of active parasitism assists to cause maximum mortality on its hosts. Depending upon the outcomes of current investigation and conferring to weevil mortality,

species of EPN can be anticipating candidates for controlling the *R. ferrugineus* larvae.

Our research demonstrated that the %age mortality of palm weevil reduced at later instars while in case of adult stage minimum or no mortality occurred upon EPNs application. Maximum mortality at earlier and minimum mortality at later instars of *R. ferrugineus* larvae was also demonstrated in a paper published by Shahina *et al*. [28]. The consequences of our research described that lower or no mortality occurs at adult stage of *R. ferrugineus* via inoculation of three EPN species. However, results showing minimum infestation of EPNs at adult stage of *R.ferrugineus* populace were also reported by Atwa *et al*. [4] which described that *H. bacteriophora*, *S. carpocapsae* and *S. feltiae* undergo minimum %age infestation of 15.3%, 8.0% and 2.0% respectively.

In the present investigation maximum and minimum mortality of the *R. ferrugineus* larvae by utilization of EPNs after 12 hours of time duration was 81% and 19.83% shown by *S. carpocapsae* and *S. feltiae* respectively. While, after a time period of 192 hours (8 days), the maximum and minimum percentage mortality of palm weevil larvae was 98.9% and 38.53% from *S. carpocapsae* and *S. feltiae* strains respectively.

5. Conclusion

In current research among three EPN species, *S. carpocapsae* resulted maximum mortality on *R. ferrugineus* larvae. In addition, active specie noticed was *H. bacteriophora* while *S. feltiae* also instigated mortality on palm weevil larvae but in current investigation it is not adequate to regulate this pest.

Our consequences prove that larvae were extremely vulnerable to the EPNs tested. All three species of EPN tested exhibited efficiency at various rates against *R. ferrugineus* populace. It was also confirmed in this study that time length plays an important role in EPNs application against the percentage mortality of *R. ferrugineus* population under laboratory conditions. In an assumption, it could be recommended that EPNs have capability to use them as bio-control agents for the management of red palm weevil. Specifically, the using prospective of both *S. carpocapsae* and *H. bacteriophora* should be explored for advance biological control examinations.

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