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Biocontrol potential of entomopathogenic fungi, nematodes and bacteria against *Rhynchophorus ferrugineus* (Olivier)

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Abstract

Background: The red palm weevil (RPW), *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae), is a serious threat to date palms across the globe, causing heavy yield losses. The pest inflicts damage to palms stem and destroys vascular system; resultantly lower the vigor and affect the growth and yield. For ecological farming system, biological control of the pest is gaining increased interest due to hosts' specificity, safety to human, animal and non-target organisms, and their compatibility to environment.

Results: In laboratory assay, *Beauveria bassiana*, *Heterorhabditis bacteriophora* and *Bacillus thuringiensis* var. *kurstaki* (*Bt-k*) alone and in combination against sixth instar larvae and adults of four distinct populations of RPW were applied. *H. bacteriophora* was more effective, followed by *B. bassiana* and *Bt-k* in alone treatments. While in combined treatments, the highest mortality was recorded for *H. bacteriophora* + *B. bassiana* combination (100% for both stages), followed by *H. bacteriophora* + *Bt-k*, (larvae 100%; adults 94.24%) and *B. bassiana* + *Bt-k* treatments (larvae: 87.01%; adults: 80.53%). Maximum rate of mycosis (larvae 85.74%; adults 69.07%), sporulation (larvae 189.22 conidia ml^{-1}; adults 164.56 conidia ml^{-1}), cadavers affected by nematodes (larvae 92.4%; adults 81.29%) and nematode production (larvae 178.78 IJs ml^{-1}; adults 153.44 IJs ml^{-1}) was observed where *B. bassiana* or *H. bacteriophora* was applied alone and the lowest (larvae 122.78 IJs ml^{-1}; adults: 103.22 IJs ml^{-1}) was recorded for *H. bacteriophora* + *B. bassiana* combination.

Conclusions: Entomopathogens can be effectively used alone and/or in integration to control RPW populations. Natural capability of entomopathogens to infect and disseminate into other hosts makes them excellent biocontrol agents to be incorporated in the IPM plan of RPW and to make palm growers confident with the use of the most promising microbial control agents.

Keywords: *Rhynchophorus ferrugineus*, Red palm weevil, *Beauveria bassiana*, *Heterorhabditis bacteriophora*, *Bacillus thuringiensis*, Entomopathogens, Potential

Background

The red palm weevil (RPW), *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae), is an invasive pest which has invaded and fully established in more than 60 countries of the world, infecting 40 palm species belonging to 23 different genera (Ashry et al. 2020). Heavy use of synthetic chemicals causes environmental damage, endangers biological diversity and exert negative impact on human and non-target organisms (Abdel-Raheem et al. 2020). Moreover, pesticides do not effectively manage the pest due to their cryptic nature and lead to the development of insecticide resistance against it. Considering these concerns, there is a growing demand to adopt alternate control measures which are

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safer to human and non-target, and compatible to environment (Mendesil et al. 2016).

Microbial entomopathogens including entomopathogenic fungi (EPFs), entomopathogenic nematodes (EPNs) and entomopathogenic bacteria (EPBs) are widely used as potential alternatives to these chemicals. EPFs are commonly found in the nature and cause epizootics in insect populations, thus play significant role in regulating insect population. EPFs from various strains of *Beauveria bassiana* (Bals.-Criv.) Vuill. and *Metarhizium anisopliae* (Metchnikoff) (Ascomycota: Hypocreales) have been found associated with RPW and among the most relevant biological agents (Faleiro 2006). *Bacillus thuringiensis* (Berliner) is another important microbial control agent which holds a prominent position among commercial chemical compounds important for agricultural insect pests. Various researchers have evaluated the pathogenic potential of *B. thuringiensis* against RPW and revealed its successful control (Manachini et al. 2009). *B. thuringiensis* causes feeding inhibition due to midgut damage in the treated larvae. EPNs have been declared an efficient entomopathogens against a variety of insects in integrated pest management program (IPM) including RPW (Manzoor et al. 2020). They are obligate parasites belonging to the families Steinernematidae and Heterorhabditidae which kill their host with the aid of mutualistic bacterium present in their intestine (Poinar 1990).

The intervention of more than one biocontrol agent can enhance the efficacy of the other partners; many studies have been conducted in this regard. The combined effect of *B. bassiana* and *B. thuringiensis* working synergistically delivered more harm to insect pests (Wraight and Ramos 2005). Similarly, the combined applications of EPNs and EPFs have been evaluated against different insect pests including RPW (Manzoor et al. 2020). Hence, integrated practices can be a hint for those, who are willing to manage RPW.

Therefore, the present study was designed to investigate the pathogenicity of *H. bacteriophora*, *B. bassiana* and *B. thuringiensis* var. *kurstaki* (*Bt-k*) against larvae and adult of RPW populations under laboratory conditions.

Methods

RPW collection and rearing

Four different populations of RPW were collected from Layyah, Dera Ismail Khan (D.I. Khan), Muzaffargarh and Rahim Yar Khan (R.Y. Khan), Punjab, Pakistan. Larvae, pupae and adults were collected from infested and fallen trees. Samples were collected and kept in separate plastic boxes assigned for a specific stage and brought to the laboratory until enough collection was done. Further multiplication for one generation was carried out in Microbial Control Laboratory, Department of Entomology, University of Agriculture, Faisalabad, Pakistan. Larvae were offered by pieces of sugarcane (*Saccharum officinarum* L.; Poales: Poaceae) stem for feeding and pupation, while shredded sugarcane pieces were offered to the adults for feeding and as a substrate for oviposition. After pupation, pupal cocoons were kept in separate plastic jars for adult emergence in an incubator. Upon emergence, weevils were shifted to the adult's jar for feeding, mating and oviposition. Colony was developed in plastic boxes ($30 \times 60 \times 60$ cm) having a lid, covered with mesh wire gauze (60 mesh size) in the middle (10 cm diameter) for aeration. Adult's diet was changed every three days and replaced sugarcane pieces were kept in separate jars for egg hatching. After egg hatching, neonates were allowed to feed for 3 days in the same set and then shifted to the sugarcane sets for feeding and pupation. Larvae were shifted to the new sugarcane sets every week until pupation. The rearing conditions were maintained at 25 ± 2 °C, 65 ± 5% RH and a 12: 12 (D: L) hrs. photoperiod (Wakil et al. 2017).

Preparation of *Bacillus thuringiensis* spore-crystal mixtures

Commercial formulation of *B. thuringiensis* var. *kurstaki* (*Bt-k*) (Dipel®) was obtained from National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. This strain was, then, subjected to sporulation by culturing in 50 ml nutrient broth media. Harvesting of culture was carried out by centrifugation at 6000 rpm for 15 min (Hernández et al. 2005). The pellets formed resultantly were washed twice in cold 1 M NaCl and thrice in sterile distilled water (SDW), re-suspended in distilled water (5 ml). From the suspension formed, 1 ml was centrifuged for 5 min at 10,000 rpm, dried for 4 h. at 37 °C and weighed (Wakil et al. 2017).

Nematode culture

The IJs of *H. bacteriophora* were obtained from the culture collection of Microbial Control Laboratory. *H. bacteriophora* was maintained in third instar *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) larvae, following the procedure of Kaya and Stock (1997).

Fungal spores

The fungal isolate of *B. bassiana* (WG-43), used in the study, was taken from the culture collection of Microbial Control Laboratory, originally isolated from dead cadaver of RPW. Fungi were sub-cultured on Sabouraud Dextrose Agar (BD, Becton, Dickisonand Company sparks, MD 21152 USA). Conidial suspension was prepared with 0.01% Tween-80 (Merck, KGaA, Darmstadt, Germany) in sterile distilled water and conidial concentration

of 1×10^7 conidia ml^{-1} determined using a Neubauer hemocytometer.

Treatment with *Beauveria bassiana*

Sixth instar RPW larvae and adults of uniform age from each population were directly immersed into the conidial suspensions for 60 and 90 s, respectively, and control was treated with aqueous solution containing 0.01% Tween-80. The treated and control insects were individually shifted to 150 ml cylindrical plastic cups (10 cups per replication), each measuring 6 cm in height with 6 cm diameter. The tops of cups were covered with fine mesh in order to avoid the insects to escape. A piece of 2×2 cm^2 artificial diet (Agar, brewer's yeast, wheat germ, corn flour, ascorbic acid, benzoic acid, amino acid-vitamin mix, chloramphenicol and nipagin) (Martín and Cabello 2006) was kept in the center of the cups for larvae, and a shredded sugarcane piece was offered to the adults. The causal agent of dead larvae or adults was confirmed by shifting the cadavers into a Petri dish lined with wet filter paper and incubating them at 25 ± 2 °C and 70 ± 5% RH for up to 15 days.

Treatment with *Bacillus thuringiensis* var. *kurstaki* (*Bt-k*)

Sixth instar RPW larvae, from each population, were individually offered with artificial diets (Martín and Cabello 2006) and mixed with the diluted spore-crystal (70 µg g^{-1}). To each larva, *Bt-k* treated diet piece of (2×2 cm^2) was provided to feed in plastic cups, and for adults, shredded sugarcane pieces were dipped in known (70 µg g^{-1}) concentration of *Bt-k* for 90 s and offered to respective population.

Treatment with *Heterorhabditis bacteriophora*

H. bacteriophora suspension was prepared at a concentration of 300 IJs ml^{-1} in glass jars, and 1 ml of suspension was poured into the 150 ml cylindrical plastic cups (6×6 cm) lined with damp Whatman filter paper. The cups were covered with a fine mesh in order to avoid the insect escape. After pouring nematodes, 30 min time was given for their even distribution on filter paper (Atwa and Hegazi 2014). A small piece of artificial diet 2×2 cm^2 was placed in middle of the cups as a food source for larvae and provided with new food every day. In each cup, one sixth instar larva from each population was placed on top of the filter paper. Same procedure was repeated for adult population and shredded sugarcane pieces were offered as food source, while control treatment received 1 ml of distilled water. Dead individuals were transferred to the modified White traps (White 1927) and left for 10 days for IJs emergence. The insects exhibiting typical odor and color (signs for nematode infestation) were considered to be killed by nematodes (Woodring and Kaya 1988).

Treatment with *Beauveria bassiana*, *Heterorhabditis bacteriophora* and *Bt-k*

For integrated application of *B. bassiana* + *Bt-k*, sixth instar larvae and adults from each population were directly immersed into the conidial suspensions for 60 and 90 s, respectively, and shifted the larvae to *Bt-k* treated diet and adults to *Bt-k* dipped shredded sugarcane pieces. For *B. bassiana* and *H. bacteriophora* combination, larvae and adults were immersed in fungal spores and released on nematode treated filter paper. While, in case of *H. bacteriophora* and *Bt-k* combination, larvae and adults were fed by *Bt-k* treated diet and *Bt-k* dipped sugarcane pieces respectively, and placed in plastic cups with nematode treated filter papers, individually. Mortality data were recorded after 7, 14 and 21 days of the treatments, each treatment was replicated 3 times with 10 individuals and whole bioassay was repeated thrice to avoid the pseudo-replication phenomenon. All the treatments were incubated at 25 ± 2 °C, 70 ± 5% RH and a 12: 12 (D: L) hrs. photoperiod.

Sporulation and nematode production

Mycosed larvae, after 14 days of incubation, were vortexed for 30 min in distilled water containing 0.01% Tween-80, and number of spores was estimated in 1 ml from the suspension using a hemocytometer. Concentration of IJs was measured in 1 ml sample from the final solution by counting the IJs with the help of a Peters' slide and microscope.

Statistical analysis

The mortality in control treatments was < 5%. It was not included in the analysis and adjusted with Abbott's formula [Corrected mortality (%) = (1 − insect population in treated unit after treatment/insect population in control unit after treatment) × 100] (Abbott 1925). The data regarding mortality, mycosis, sporulation and percentage of larvae and adults affected and nematode production were subjected to factorial analysis of variance (ANOVA) in Minitab (Minitab 2003). Means were separated using Tukey's Kramer test (HSD) (Sokal and Rohlf 1995) at 5% significance level to evaluate the impact of treatments on mortality and studied parameters.

Results

Mortality of larvae and adult

The results of present study revealed that the larval and adult mortality was significantly affected by the main effects and their associated interactions, except Treatment × Population, Exposure interval × Population and Treatment × Exposure interval × Population (Table 1). There was non-significant difference in mortalities of

Table 1 ANOVA parameters for the main effects and associated interactions for mortality rates of *Rhynchophorus ferrugineus* larvae and adults

S.O.V		Larvae		Adult	
	df	*F*	*P*	*F*	*P*
Treatment	5	454.81	≤ 0.05	437.56	≤ 0.05
Exposure interval	2	1546.71	≤ 0.05	1099.79	≤ 0.05
Population	4	25.54	≤ 0.05	17.88	≤ 0.05
Treatment × Exposure interval	10	19.98	≤ 0.05	26.11	≤ 0.05
Treatment × Population	20	0.58	0.92	0.64	0.88
Exposure interval × Population	8	0.93	0.49	1.24	0.27
Treatment × Exposure interval × Population	40	0.73	0.89	0.41	0.99
Error	550	–	–	–	–
Total	647	–	–	–	–

larvae and adults ($P \leq 0.05$) among the tested populations after 7 days of exposure, except *Bt-k* + *H. bacteriophora* for larvae (Table 2). Similarly, non-significant difference in mortalities ($P \leq 0.05$) was observed at 14 days post exposure, except *Bt-k* + *B. bassiana, Bt-k* + *H. bacteriophora*, and *B. bassiana* + *H. bacteriophora* for larvae, and *B. bassiana* + *H. bacteriophora* for adults (Table 3). Likewise, significant difference was also observed in mortalities for *B. bassiana* and *H. bacteriophora* in sole application for larvae, and *Bt-k* + *B. bassiana* and *Bt-k* + *H. bacteriophora* for adults in all populations after 21 days of application (Table 4).

Significant difference was observed among all the treatments for tested RPW populations after 7 days of exposure. The highest larval and adult mortality was recorded in combined treatments of *B. bassiana* + *H. bacteriophora* for all populations (Larvae: Layyah 51.68%, R.Y. Khan 46.82%, D.G. Khan 42.19%, Muzaffargarh 39.40%; Adults: Layyah 39.73%, R.Y. Khan 36.08%, D.G. Khan 32.37%, Muzaffargarh 30.89%), followed by *Bt-k* + *H. bacteriophora*, *Bt-k* + *B. bassiana,* and sole application

Table 2 Mortality percentage of *Rhynchophorus ferrugineus* populations treated with *Bt-k* (70 µg g^{-1}), *Beauveria bassiana* (1×10^7 conidia ml^{-1}) and *Heterorhabditis bacteriophora* (300 IJs ml^{-1}) applied alone or in combination after 7 days of exposure

Stage	Treatments	Motility%					
		Layyah	D.G. Khan	Muzaffargarh	R.Y. Khan	*F*	*P*
Larvae	*Bt-k*	9.47 ± 0.78e	6.47 ± 0.55d	6.08 ± 0.78d	7.93 ± 0.71e	0.38	0.77
	Bb	13.48 ± 1.18de	10.47 ± 1.12 cd	8.39 ± 1.01 cd	11.68 ± 1.16de	0.30	0.82
	EPN	25.57 ± 1.39 cd	21.57 ± 1.38bc	19.58 ± 1.17bc	23.45 ± 1.13 cd	0.62	0.60
	Bt-k + Bb	32.28 ± 1.61bc	28.36 ± 1.46ab	25.06 ± 1.30b	30.17 ± 1.65bc	1.28	0.29
	Bt-k + EPN	45.37 ± 2.15ab	33.73 ± 1.50ab	28.08 ± 1.57ab	42.11 ± 2.36ab	6.46	≤ 0.05
	Bb + EPN	51.68 ± 2.34a	42.19 ± 2.51a	39.40 ± 2.14a	46.82 ± 2.54a	2.05	0.12
	F	24.8	30.5	18.8	14.4	–	–
	P	≤ 0.05	≤ 0.05	≤ 0.05	≤ 0.05	–	–
Adults	*Bt-k*	6.54 ± 0.70d	4.69 ± 0.49d	4.02 ± 0.53c	5.46 ± 0.74d	0.24	0.87
	Bb	10.46 ± 1.12d	8.41 ± 0.87 cd	7.56 ± 1.04bc	9.29 ± 1.09 cd	0.16	0.92
	EPN	19.18 ± 1.24 cd	14.90 ± 1.01bcd	13.22 ± 1.35bc	16.51 ± 1.21 cd	0.72	0.54
	Bt-k + Bb	24.70 ± 1.65bc	21.24 ± 1.96abc	19.69 ± 1.56ab	22.06 ± 1.56bc	0.31	0.81
	Bt-k + EPN	35.41 ± 2.35ab	28.84 ± 2.11ab	27.09 ± 1.75b	31.52 ± 2.13ab	0.90	0.45
	Bb + EPN	39.73 ± 2.09a	32.37 ± 2.03a	30.89 ± 2.05a	36.08 ± 2.45a	1.38	0.26
	F	19.2	8.90	12.4	14.4	–	–
	P	≤ 0.05	≤ 0.05	≤ 0.05	≤ 0.05	–	–

Mortality% followed by the same letter within each treatment and insect populations are not significantly different; HSD test $P \leq 0.05$ (*Bt-k*: *Bacillus thuringiensis* var. *kurstaki*, Bb: *Beauveria bassiana*, EPN: *Heterorhabditis bacteriophora*)

Table 3 Mortality percentage of *Rhynchophorus ferrugineus* treated with *Bt-k* (70 µg g^{-1}), *Beauveria bassiana* (1×10^7 conidia ml^{-1}) and *Heterorhabditis bacteriophora* (300 IJs ml^{-1}) applied alone or in combination after 14 days of exposure

Stage	Treatments	Insect populations					
		Layyah	D.G. Khan	Muzaffargarh	R.Y. Khan	*F*	*P*
Larvae	*Bt-k*	22.50 ± 1.58c	20.67 ± 1.39c	17.70 ± 1.08c	19.50 ± 1.23c	0.43	0.73
	Bb	31.02 ± 1.81c	27.76 ± 1.85c	23.39 ± 1.92c	25.64 ± 1.08c	0.59	0.62
	EPN	60.38 ± 2.71b	55.66 ± 2.44b	49.22 ± 2.65b	51.57 ± 2.18b	1.38	0.26
	Bt-k + Bb	65.24 ± 3.10b	59.10 ± 2.18b	52.55 ± 2.77b	55.09 ± 3.07b	3.39	≤ 0.05
	Bt-k + EPN	89.66 ± 3.26a	83.10 ± 3.72a	74.57 ± 3.14a	78.42 ± 3.87a	4.07	≤ 0.05
	Bb + EPN	97.37 ± 2.73a	91.55 ± 3.27a	82.68 ± 3.86a	86.17 ± 3.03a	3.67	≤ 0.05
	F	86.2	73.1	46.3	60.3	–	–
	P	≤ 0.05	≤ 0.05	≤ 0.05	≤ 0.05	–	–
Adults	*Bt-k*	17.40 ± 1.46c	15.78 ± 1.04c	12.01 ± 0.88c	13.76 ± 1.07c	0.73	0.54
	Bb	24.07 ± 1.50c	21.85 ± 1.28c	17.85 ± 1.33 cd	20.52 ± 1.32c	0.37	0.77
	EPN	45.06 ± 2.48b	40.40 ± 1.65b	34.54 ± 2.03bc	37.62 ± 1.99b	0.93	0.43
	Bt-k + Bb	52.54 ± 2.49b	48.01 ± 2.14b	39.98 ± 2.19b	43.27 ± 2.75b	2.56	0.07
	Bt-k + EPN	72.37 ± 3.21a	67.77 ± 3.10a	61.64 ± 2.93a	65.80 ± 2.57a	1.48	0.23
	Bb + EPN	81.29 ± 3.37a	75.74 ± 3.34a	64.43 ± 3.06a	70.18 ± 2.28a	3.87	≤ 0.05
	F	50.5	42.1	27.9	41.8	–	–
	P	≤ 0.05	≤ 0.05	≤ 0.05	≤ 0.05	–	–

Mortality% followed by the same letter within each treatment and insect populations are not significantly different; HSD test $P \leq 0.05$ (*Bt-k*: *Bacillus thuringiensis* var. *kurstaki*, Bb: *Beauveria bassiana*, EPN: *Heterorhabditis bacteriophora*)

Table 4 Mortality percentage of *Rhynchophorus ferrugineus* treated with *Bt-k* (70 µg g^{-1}), *Beauveria bassiana* (1×10^7 conidia ml^{-1}) and *Heterorhabditis bacteriophora* (300 IJs ml^{-1}) applied alone or in combination after 21 days of exposure

Stage	Treatments	Insect populations					
		Layyah	R.Y. Khan	Muzaffargarh	D.G. Khan	*F*	*P*
Larvae	*Bt-k*	58.36 ± 4.55d	54.66 ± 4.46c	46.86 ± 4.53d	49.78 ± 2.45c	1.55	0.22
	Bb	72.63 ± 3.88c	66.79 ± 3.45c	57.27 ± 2.75 cd	55.55 ± 2.92c	6.04	≤ 0.05
	EPN	84.54 ± 2.71bc	81.06 ± 3.14b	70.45 ± 3.19bc	73.92 ± 2.74b	4.75	≤ 0.05
	Bt-k + Bb	87.01 ± 3.04b	82.17 ± 2.90b	75.77 ± 4.75b	78.24 ± 2.39b	2.09	0.12
	Bt-k + EPN	100.00 ± 0.00a	98.41 ± 1.58a	93.35 ± 3.06a	95.57 ± 2.85a	1.75	0.17
	Bb + EPN	100.0 ± 0.00a	100.0 ± 0.00a	100.0 ± 0.00a	100.0 ± 0.00a	–	–
	F	29.8	34.9	35.5	69.0	–	–
	P	≤ 0.05	≤ 0.05	≤ 0.05	≤ 0.05	–	–
Adults	*Bt-k*	39.04 ± 2.41d	32.56 ± 3.80d	26.79 ± 3.68f	30.74 ± 2.96f	2.45	0.08
	Bb	47.55 ± 2.60d	43.07 ± 4.15d	38.13 ± 4.09e	36.12 ± 3.31e	2.03	0.12
	EPN	61.13 ± 3.81c	58.15 ± 3.25c	52.37 ± 2.20d	54.35 ± 1.82d	1.83	0.16
	Bt-k + Bb	80.53 ± 2.66b	76.69 ± 3.11b	67.96 ± 3.92c	71.04 ± 3.18c	2.99	≤ 0.05
	Bt-k + EPN	94.24 ± 2.28a	90.03 ± 2.82a	81.27 ± 2.28b	86.48 ± 1.46b	5.88	≤ 0.05
	Bb + EPN	100.0 ± 0.00a	100.0 ± 0.00a	100.0 ± 0.00a	100.0 ± 0.00a	–	–
	F	95.3	70.9	80.5	130	–	–
	P	≤ 0.05	≤ 0.05	≤ 0.05	≤ 0.05	–	–

Mortality% followed by the same letter within each treatment and insect populations are not significantly different; HSD test $P \leq 0.05$ (*Bt-k*: *Bacillus thuringiensis* var. *kurstaki*, Bb: *Beauveria bassiana*, EPN: *Heterorhabditis bacteriophora*)

of *H. bacteriophora, B. bassiana* and *Bt-k* (Table 2). The laboratory population was more susceptible, followed by D.G. Khan, R.Y. Khan and Muzaffargarh population at all exposure intervals. Likewise, after 14 and 21 days post-exposure, combined treatment of *B. bassiana* + *H. bacteriophora* exhibited the highest larval and adult mortality

rates, while the lowest one was recorded at *Bt-k* in individual applications for all RPW populations (Tables 3, 4). After last count, *B. bassiana* + *H. bacteriophora* treatment exhibited 100% larval and adult mortality for all the populations, while *Bt-k* + *H. bacteriophora* exhibited 100% larval mortality in laboratory population.

Mycosis and sporulation

Significant difference in the mycosis was observed for all RPW populations for both larvae (D.G. Khan: $F_{2, 26} = 11.2$, $P < 0.01$; Layyah: $F_{2, 26} = 21.6$, $P < 0.01$; Muzaffargarh: $F_{2, 26} = 30.8$, $P < 0.01$; R.Y. Khan: $F_{2, 26} = 13.2$, $P < 0.01$) and adults (D.G. Khan: $F_{2, 26} = 25.8$, $P < 0.01$; Layyah: $F_{2, 26} = 7.20$, $P < 0.01$; Muzaffargarh: $F_{2, 26} = 30.8$, $P < 0.01$; R.Y. Khan: $F_{2, 26} = 13.6$, $P < 0.01$). Maximum mycosed larvae (85.74%) and adults (69.07%) were observed in treatments, where *B. bassiana* was applied alone against larvae and adults, respectively, in laboratory population (Fig. 1a, b) however, low rate of mycosis was observed in the treatments where *H. bacteriophora* and *B. bassiana* were applied in combined manners. Similarly, significant difference in the sporulation was observed for all RPW populations for both larvae (D.G. Khan: $F_{2, 26} = 62.7$, $P < 0.01$; Layyah: $F_{2, 26} = 259$, $P < 0.01$; Muzaffargarh: $F_{2, 26} = 140$, $P < 0.01$; R.Y. Khan: $F_{2, 26} = 132$, $P < 0.01$) and adults (D.G. Khan: $F_{2, 26} = 163$, $P < 0.01$; Layyah: $F_{2, 26} = 197$, $P < 0.01$; Muzaffargarh: $F_{2, 26} = 155$, $P < 0.01$; R.Y. Khan: $F_{2, 26} = 138$, $P < 0.01$). Maximum sporulating larvae and (189.22 conidia ml^{-1}) adults (164.56 conidia ml^{-1}) were observed in treatments, where *B. bassiana*

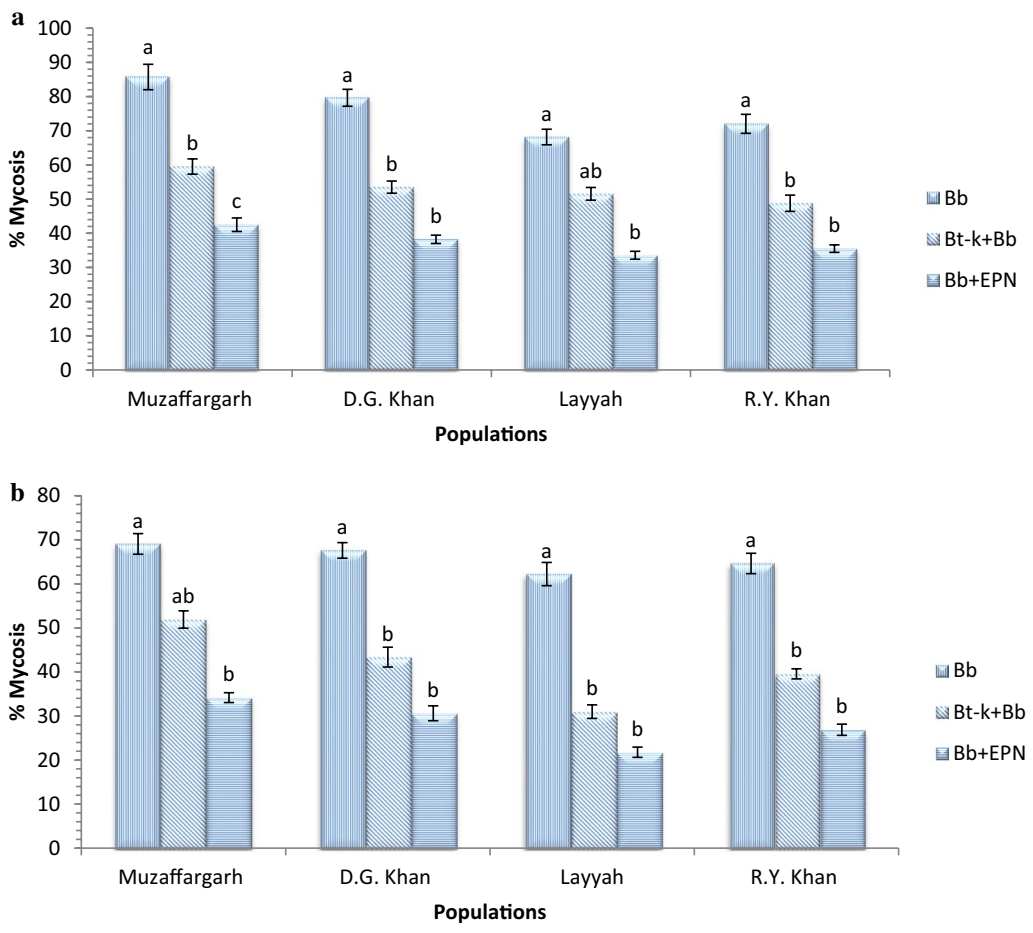

Fig. 1 **a** Mycosis percentage in *Rhynchophorus ferrugineus* larvae treated with *Bt-k* (70 µg g^{-1}), *Beauveria bassiana* (1×10^7 conidia ml^{-1}) and *Heterorhabditis bacteriophora* (300 IJs ml^{-1}) applied alone or in combination. Percentage mycosis followed by the same letter within each treatment are not significantly different; HSD test $P \leq 0.05$ ((*Bt-k*: *Bacillus thuringiensis* var. *kurstaki*, Bb: *Beauveria bassiana*, EPN: *Heterorhabditis bacteriophora*). **b** Mycosis percentage in *Rhynchophorus ferrugineus* adults treated with *Bt-k* (70 µg g^{-1}), *Beauveria bassiana* (1×10^7 conidia ml^{-1}) and *Heterorhabditis bacteriophora* (300 IJs ml^{-1}) applied alone or in combination. Percentage mycosis followed by the same letter within each treatment are not significantly different; HSD test $P \leq 0.05$ (*Bt-k*: *Bacillus thuringiensis* var. *kurstaki*, Bb: *Beauveria bassiana*, EPN: *Heterorhabditis bacteriophora*)

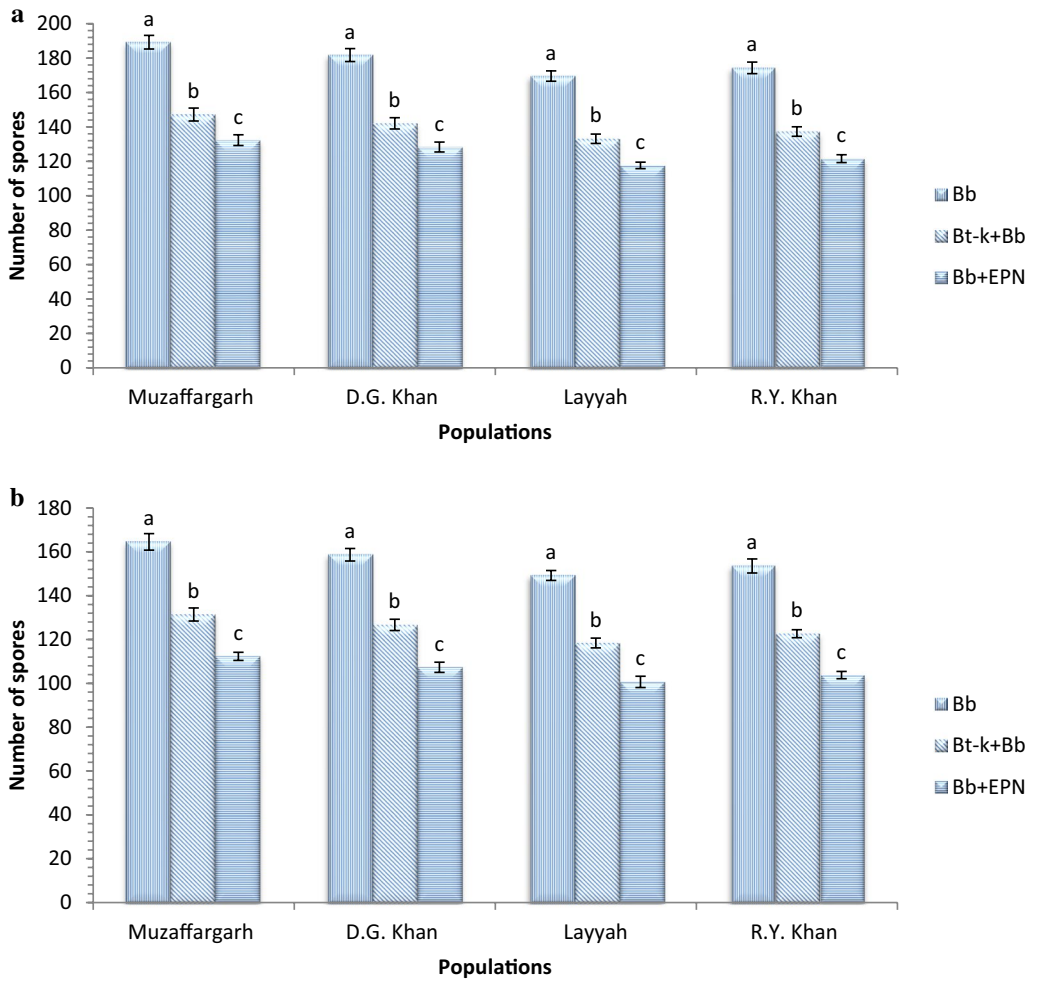

Fig. 2 **a** Sporulation (conidia ml^{-1}) in *Rhynchophorus ferrugineus* larvae treated with *Bt-k* (70 µg g^{-1}), *Beauveria bassiana* (1 × 10^{7} conidia ml^{-1}) and *Heterorhabditis bacteriophora* (300 IJs ml^{-1}) applied alone or in combination. Treatments followed by the same letter within each treatment are not significantly different; HSD test $P \leq 0.05$ (*Bt-k*: *Bacillus thuringiensis* var. *kurstaki*, Bb: *Beauveria bassiana*, EPN: *Heterorhabditis bacteriophora*). **b** Sporulation (conidia ml^{-1}) in *Rhynchophorus ferrugineus* adults treated with *Bt-k* (70 µg g^{-1}), *Beauveria bassiana* (1 × 10^{7} conidia ml^{-1}) and *Heterorhabditis bacteriophora* (300 IJs ml^{-1}) applied alone or in combination. Treatments followed by the same letter within each treatment are not significantly different; HSD test $P \leq 0.05$ (*Bt-k*: *Bacillus thuringiensis* var. *kurstaki*, Bb: *Beauveria bassiana*, EPN: *Heterorhabditis bacteriophora*)

was applied alone against laboratory population (Fig. 2a, b); however, low rate of sporulation was observed in the treatments where *H. bacteriophora* and *B. bassiana* were applied in combined manners. Similar pattern in mycosis and sporulation was observed in other RPW populations.

Insects affected by EPN and their production

Significant difference in infection rate by nematodes was observed for larvae (D.G. Khan: $F_{2,\ 26} = 17.19$, $P < 0.01$; Layyah: $F_{2,\ 26} = 29.9$, $P < 0.01$; Muzaffargarh: $F_{2,\ 26} = 11.2$, $P < 0.01$; R.Y. Khan: $F_{2,\ 26} = 15.0$, $P < 0.01$) and adults (D.G. Khan: $F_{2,\ 26} = 7.98$, $P < 0.01$; Layyah: $F_{2,\ 26} = 10.6$, $P < 0.01$; Muzaffargarh: $F_{2,\ 26} = 9.29$, $P < 0.01$; R.Y. Khan: $F_{2,\ 26} = 8.27$, $P < 0.01$). Maximum lethality in affected individuals was 92.40 and 81.29% in larvae and adults, respectively (Fig. 3a, b). Likewise, significant difference was recorded in nematode production both for larvae (D.G. Khan: $F_{2,\ 26} = 34.0$, $P < 0.01$; Layyah: $F_{2,\ 26} = 85.1$, $P < 0.01$; Muzaffargarh: $F_{2,\ 26} = 31.0$, $P < 0.01$; R.Y. Khan: $F_{2,\ 26} = 62.4$, $P < 0.01$) and adults (D.G. Khan: $F_{2,\ 26} = 25.4$, $P < 0.01$; Layyah: $F_{2,\ 26} = 46.8$, $P < 0.01$; Muzaffargarh: $F_{2,\ 26} = 17.5$, $P < 0.01$; R.Y. Khan: $F_{2,\ 26} = 53.3$, $P < 0.01$). Maximum number of nematode productions, on white trap in larvae (178 IJs ml^{-1}) and adults (153 IJs ml^{-1}) (total volume 10 ml), was observed in treatments where *H. bacteriophora* was applied alone against larvae and adults in the laboratory population (Fig. 4a, b). However, low infection rate and nematode's production were recorded

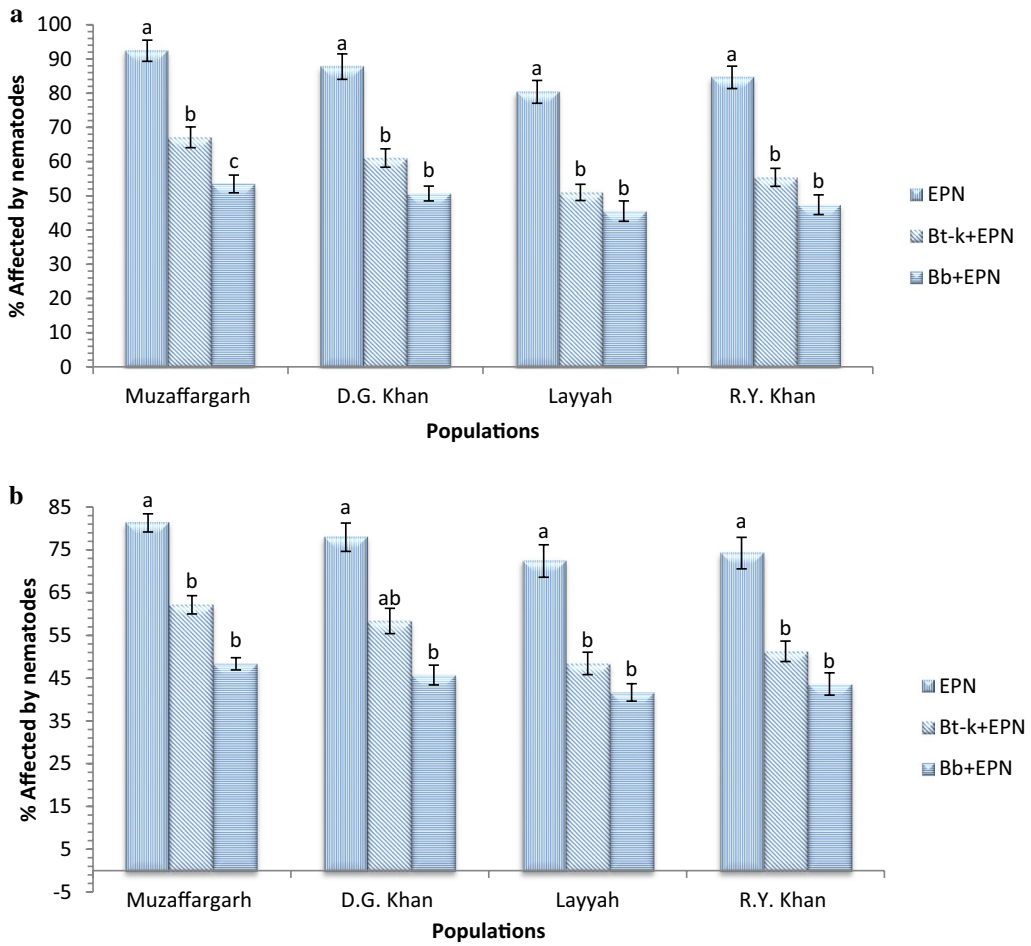

Fig. 3 **a** *Rhynchophorus ferrugineus* larvae affected by *Heterorhabditis bacteriophora* treated with *Bt-k* (70 µg g^{-1}), *Beauveria bassiana* (1×10^7 conidia ml^{-1}) and *Heterorhabditis bacteriophora* (300 IJs ml^{-1}) applied alone or in combination. Treatments followed by the same letter within each treatment are not significantly different; HSD test $P \leq 0.05$ (*Bt-k*: *Bacillus thuringiensis* var. *kurstaki*, Bb: *Beauveria bassiana*, EPN: *Heterorhabditis bacteriophora*). **b** *Rhynchophorus ferrugineus* adults affected by *Heterorhabditis bacteriophora* treated with *Bt-k* (70 µg g^{-1}), *Beauveria bassiana* (1×10^7 conidia ml^{-1}) and *Heterorhabditis bacteriophora* (300 IJs ml^{-1}) applied alone or in combination. Treatments followed by the same letter within each treatment are not significantly different; HSD test $P \leq 0.05$ (*Bt-k*: *Bacillus thuringiensis* var. *kurstaki*, Bb: *Beauveria bassiana*, EPN: *Heterorhabditis bacteriophora*)

in the treatments where *H. bacteriophora* and *B. bassiana* were integrated. Similar trend was recorded for the other RPW populations.

Discussion

Based on the results of the present study, the integrated use of entomopathogens is effective, but the outcomes were highly influenced by the combination of agents (*Bt-k* plus *B. bassiana*, *Bt-k* plus *H. bacteriophora* or *B. bassiana* plus *H. bacteriophora*), host stage and the exposure interval. The integration of entomopathogenic nematodes + fungi and bacteria is entirely new approach to control RPW larvae and adults as the pathogens exhibited virulence against both developmental stages of RPW. However, when combined either agent, the pathogenicity was enhanced, except larvae at last count. Numerous studies have documented sole and integrated applications of entomopathogens against number of insect pests (Usman et al. 2020). The entomopathogens, that curtail RPW infestations, are more effective in managing weevil population than chemical insecticides and fumigants (El-Sufty et al. 2011).

In this study, the combined treatments of *B. bassiana* + *Bt-k* exhibited enhanced larval and adult mortality than their individual applications. Integrated application of *B. bassiana* + *B. thuringiensis* acted synergistically and weakened the insect immune response system which allows entomopathogens to inflict high mortality (Wakil

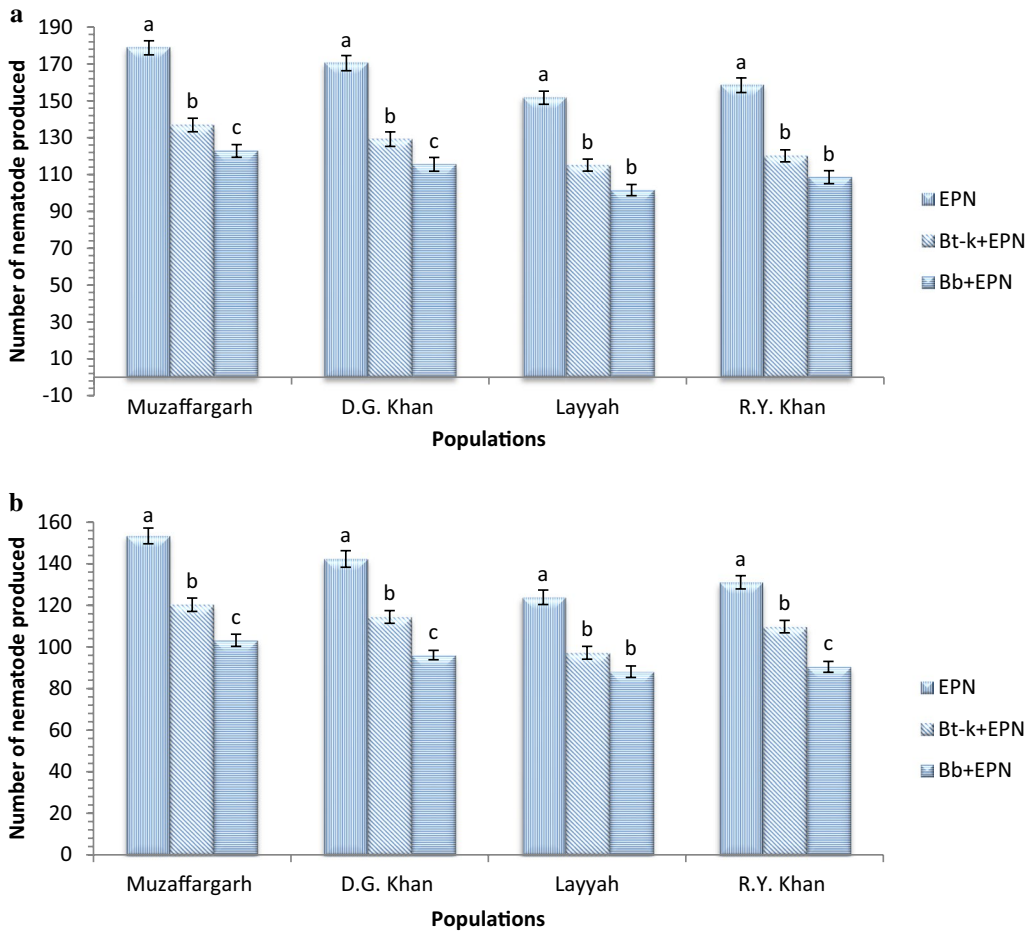

Fig. 4 **a** Nematode production (IJs ml^{-1}) in larvae of *Rhynchophorus ferrugineus* treated with *Bt-k* (70 µg g^{-1}), *Beauveria bassiana* (1×10^7 conidia ml^{-1}) and *Heterorhabditis bacteriophora* (300 IJs ml^{-1}) applied alone or in combination. Treatments followed by the same letter within each treatment are not significantly different; HSD test $P \leq 0.05$ (*Bt-k*: *Bacillus thuringiensis* var. *kurstaki*, Bb: *Beauveria bassiana*, EPN: *Heterorhabditis bacteriophora*). **b** Nematode production (IJs ml^{-1}) in adults of *Rhynchophorus ferrugineus* treated with *Bt-k* (70 µg g^{-1}), *Beauveria bassiana* (1×10^7 conidia ml^{-1}) and *Heterorhabditis bacteriophora* (300 IJs ml^{-1}) applied alone or in combination. Treatments followed by the same letter within each treatment are not significantly different; HSD test $P \leq 0.05$ (*Bt-k*: *Bacillus thuringiensis* var. *kurstaki*, Bb: *Beauveria bassiana*, EPN: *Heterorhabditis bacteriophora*)

et al. 2020). When fungal spores gain access to the insect gut, it boosts the infection of *Bt* spores, consequently help each other in retardation of normal physiological functions of an insect host. Inversely, the *Bt* treatments lead the gut to septicemia causing the insect to stop feeding, and weakening the host immune system. This will favor the *B. bassiana* to work efficiently with very low resistant of the host immune system thereby increasing mortality. These findings are in line with Allee et al. (1990) who reported the synergistic interaction of *B. bassiana* and *Bt* against grubs of Colorado potato beetle.

We have observed the highest mortality by EPNs in combined treatments with *Bt-k* and *B. bassiana* of both larvae and adults. While, in experiment conducted by Koppenhöfer and Kaya (1997), the results did not indicate a potential synergism between *Bt* var. *kurstaki* and *H. bacteriophora*. This might be attributed to the inadequate weather conditions in the days after nematode application.

Obtained results are also in accordance with the findings of many researchers who reported additive or synergistic interactions among nematodes and fungi (Manzoor et al. 2020). Contrarily, Shapiro-Ilan et al. (2004) found antagonism between EPNs and *P. fumosoroseus*. Such antagonism may be due to pathogen interactions prior to or during infection. The findings also suggested that percent mycosis and sporulation were high in treatment where *B. bassiana* was

applied alone. Similar results were reported by Tefera and Pringle (2003) who determined a high mycosis and sporulation on larvae of *Chilo partellus* (F.) (Pyralidae: Lepidoptera). In case of combined applications, all insects died quickly and fungus sporulated in a small number of cadavers and created few conidia. Similar results were also reported by Malik et al. (2016) who reported high mycosis and sporulation against RPW larvae. Similar phenomenon could be attributing the higher IJs production in sole treatment of nematodes.

A good knowledge of biological parameters of RPW and, most importantly, the interaction among entomopathogens could play a key role to expand RPW-IPM programs. This calls for the isolation and identification of more virulent strains of entomopathogens. Moreover, the field evaluation of these substances in combined manners can provide substantial information and help in developing new strategies for IPM based crop production systems (Manachini et al. 2011).

Conclusions

The present study revealed that *B. bassiana, Bt-k* and *H. bacteriophora* can effectively kill the RPW larvae and adults. They also exert the detrimental effect on their growth and development which can be used effectively against this pest. Moreover, mycosis and nematode production is the peculiar characters of EPFs and EPNs that can cause secondary infection and novel mode of actions of these pathogenic agents can become a useful component in an IPM program against RPW.

Abbreviations

Bt-k: *Bacillus thuringiensis* Var. *kurstaki*; D: Day; D.G. Khan: Dera Ghazi Khan; EPF: Entomopathogenic fungi; EPN: Entomopathogenic nematodes; IJs: Infective juveniles; IPM: Integrated pest management; L: Light; Ml: Milliliter; RPW: Red palm weevil; R.Y. Khan: Rahim Yar Khan.

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Authors' contributions

All authors equally participated in this study. WW conceived and designed the study. MAQ and MY conducted the research and prepared first draft. SA performed statistical analysis and prepared graphs. AS, MAA and MS supervised, reviewed and edited MS for final submission. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval

Not applicable.

Consent of publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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