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Synergistic Effect of Entomopathogenic Fungi and Bacteria against Pulse Beetle, *Callosobruchus chinensis*

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Authors' Contribution
MI Conducted research. FAS conceptualized and supervised research. RM and MKF provided research materials and analysed the data. IB proofread the manuscript. FN and MUR facilitated in conducting pathological aspects of research.

Key words
***Cicer arietinum*, *Callosobruchus chinensis*, Entomopathogenic fungi, Entomopathogenic bacteria, DEBBM, Mortality.**

ABSTRACT

Callosobruchus chinensis is one of the most destructive insect pests of *Cicer arietinum* (Chickpea) in storages and renders grains unfit for human consumption. In the present study, synergistic effects of entomopathogenic fungi (EPF), *Beauveria bassiana* and *Metarhizium anisopliae* and entomopathogenic bacteria (EPB), *Photorhabdus temperata* and *Xenorhabdus nematophila* were studied as bio-control agents to manage this pest. In addition, percent conidial germination and % mortality of *C. chinensis* were also evaluated when *B. bassiana* was used with DEBBM. The minimum number of eggs (0.66 grain^{-1}), number of holes (1 grain^{-1}), number of F_1 new emerged (5.6 jar^{-1}), days to 100 percent mortality F_1 (3), weight loss (4%), damage (5%) whereas the maximum inhibition rate of 85% and percent mortality of *C. chinensis* were observed in synergistic concentration of EPB as compared to EPF. Synergistic concentration (1×10^8) of bacteria gave the best results against *C. chinensis* as compared to that of entomopathogenic fungi. The maximum percent conidial germination (90%) of *B. bassiana* and percent mortality (50.40±2.20a), (79.70±0.28a) and (90.76±0.56a) of *C. chinensis* were observed when DEBBM was synergized with *B. bassiana* after seven, fourteen and twenty one days, respectively.

INTRODUCTION

Chickpea ranks 2nd in area under cultivation and 3rd in production among pulses in the world (Iqbal *et al*., 2018). In Pakistan, annual production of 0.5m tonnes of dry seed is obtained from an area of around 1m hectares (Iqbal *et al*., 2018). In storages, it has severe post harvest losses due to *Callosobruchus chinensis* (Ahmed *et al*., 2011), which causes about 10% damage and renders grains unfit for human consumption (Aslam, 2004).

For the control of stored grain insect pests, grain protectants and fumigants have been used for the last many years. However, these chemicals lost effectiveness due to resistance developed in insects and presence of residual effects is the other demerit posing risks to human health and the environment (Rajendran and Sriranjini, 2008). Botanicals have also been used as safe grain protectants against stored grain insect pests (Bakkali *et al*., 2008) but also faced resistance issues inviting the other safer approaches like entomopathogens to manage insect pests.

Entomopathogenic fungi (EPF) are widely distributed with both restricted and wide host ranges. They have different biocontrol potentials against arthropods insects and plant pathogenic fungi. They are among the first organisms to be used for the biological control of pests. Target insect pests of *M. anisopliae* are stored grain insect pests namely *Callosobruchus* sp., and others like cockroaches and locusts (Nabaei *et al*., 2012). Target insect pests of *B. bassiana* are the stored insect pests, *Callosobruchus* sp., *Tribolium castaneum*, *Sitophilus granarius* and *Oryzaephilus surinamensis* and others like mites and white flies (Khashaveh *et al*., 2008; Nabaei *et al*., 2012).

Entomopathogenic bacteria (EPB), namely *P. temperata* and *X. nematophila* are symbiotic in nature. Significant pathogenicity has been observed between these two bacteria against the stored insect pests. Main target insect pests of these bacteria are stored grain insect pests namely red flour beetle and other insects like *Spodoptera litura*, diamond back moth (Sony and Kim, 2010; Jung and Kim, 2006).

The objectives of the proposed research were to investigate synergistic effect of EPF (*B. bassiana* and *M. anisopliae*) and EPB (*P. temperata* and *X. nematophila*). In addition DEBBM (mixture of DE (Diatomaceous Earth) and BBM (Bitterbarkomycin, Chinese plant extract))

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effectiveness as inert material was also studied.

MATERIALS AND METHODS

The study was carried out at Stored Grain Pests Research Lab of Entomology Department of Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, during 2017. Infested samples of stored chickpea grains were collected from different research stations including National Agriculture Research Council (NARC) Islamabad, Pakistan. *C. chinensis* culture was maintained in an incubator at temperature of 30±2°C and 70±5% relative humidity in the Department of Entomology, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, Pakistan and was named as 'Pulse Beetle Rearing Cell' during 2017. For bioassays, chickpea cultivar NOOR-2009 was obtained from NARC, Islamabad. Agtoxin was applied for fumigation to kill already existing insect pests (Shaheen *et al*., 2006). EPF namely *B. bassiana* isolate (DEBI 005) and *M. anisopliae* isolate (DEMI 001) and EPB *P. temperata* isolate (ANU101) and *X. nematophila* isolate (K1) were obtained from Korean Agricultural Culture Collection (KACC), NAC, RDA, Suwon, 441-707, Korea.

Initially the culture was grown in Potato Dextrose Agar (PDA) at 25°C at 200 rpm for two weeks, and then it was multiplied in Potato Dextrose Broth medium to count the number of conidia/ml. The conidia/ml was grown on PDA medium. Later on then conidia/ml was counted by haemocytometer at 24 h interval (Tuan *et al*., 2009). Then petri dishes were kept in incubator at a temperature of 28±1°C and relative humidity of 75±5% for fungi mass culturing. After drying the culture in oven, rubber scalpel was used to harvest the conidia from culture. Fungal concentrations of 1×10^8 of both fungi were synergized by adding the sterile 0.02% (Tween 80) in sterile distilled (purified) water (Atif *et al*., 2012).

Initially, the culture was streaked on nutrient agar (NA) plates at 25°C for 4-6 days. Culture was purified by re-streaking single colony on NA. Purified culture was multiplied in nutrient broth at 200 rpm for two to three days. To count/optimize the (colony forming units) cfu's per unit volume serial dilution method was employed for drawing dilution curve between optical density (OD) and cfu's. Bacterial dilutions of 1×10^8 of both bacteria were prepared (Atif *et al*., 2012). Each of the EPF and EPB were applied @ 2ml per jar against *C. chinensis.*

Experiment No. 1

In each jar, 50g of chickpea grains was put in plastic jars. The jars were covered with muslin cloth tightened and then placed in an incubator at 30ºC. Ten pairs of *C. chinensis* were released into each jar. Best concentration *viz*. 1×10^8 (conidia ml^{-1}) of both bacteria and fungi were prepared for experiment. Parameters of study includes number of eggs, number of holes, number of F_1 progeny adults emerged, percent inhibition rate (% IR), days to 100% mortality of F_1 emerged, weight loss (%), and percent damage.

The formulae used were (Shaheen *et al*., 2006) as follows:

$$\%IR = \frac{(C_n - T_n)}{C_n} \times 100$$

Where, C_n is the number of newly emerged adults in untreated jar (control) and T_n is the number of newly emerged adults in treated jar.

$$\text{WL (\%)} = \frac{\text{Initial wt.} - \text{Wt. of damaged grains}}{\text{Initial wt.}} \times 100$$

$$\%\text{damage} = \frac{\text{No. of damaged grains}}{\text{No. of total grain}} \times 100$$

Where, WL is weight loss and wt. is weight

Experiment No. 2

Mortality of *C. chinensis* was determined in treated and untreated 50g of stored chickpea grains in each petri plate 07cm diameter ((38.5 cm^2), which had filter paper (Whatman No. 1). The concentration of bacteria and fungi *viz*., 1×10^8 (conidia/ml) were applied @ 2ml on grains. Each treatment had 3 replications. The mortality of *C. chinensis* due to concentration of both EPF and EPB was observed after 24, 48 and 72 h. Ten beetles of *C. chinensis* were released. Petri-plates were placed in an incubator at 30ºC.

Experiment No. 3

DE component of DEBBM belongs to a group of freshwater DEs composed of amorphous SiO2 (89%), Al_2O_3 (4%), Fe_2O_3 (1.7%), CaO (1.4%), MgO + K_2O (less than 1%), H_2O (3%). Median particle size is 10 mm, specific gravity 2.2, surface area 35.7 m^2g^{-1}, pH 8 and 0.1% crystalline silica. BBM (Bitterbarkomycin, Chinese plant extract) is a polyol ester, extracted from the roots of the plant, *Celastrus angulatus.* Experiment conducted at temperatures of 25, 30 and 35°C (70% RH). 50 g of grain samples placed in plastic jars. Following concentrations were used: (i) D1 (20 mg of DEBBM), (ii) D2 (40mg of DEBBM), (iii) B1 (6.69×10^5 conidia/kg), (iv) B2 (6.69×10^5 conidia/kg), (v) B3 (6.69×10^5 conidia/kg), (vi) D1+B1, (vii) D1+B2, (viii) D1+B3, (ix) D2+B1, (x) D2+B2 and (xi) D2+B3.

Both *B. bassiana* and DEBBM were thoroughly

mixed and shook manually for 2 min. Twenty adults of *C. chinensis* were released into each jar. Adult mortality was recorded after 7, 14 and 21 days of treatments application.

Statistical analysis

SPSS 16.0 for Windows program was used for one factor analysis. Duncan's multiple range test (DMRT) was applied to all the means. Moreover the graphical work was done using Microsoft excel programme.

Table I.- Number of eggs (Mean ± SEM) per grain laid by *C. chinensis* and number of hols/grain in chickpea grains treated with different synergistic concentrations of *B. bassiana*+*M. anisopliae* and *P. temperata*+*X. nematophila*.

S No.	Concentrations 1×10^8 (conidia/ml)	No. of eggs/ grain	No. of holes/ grain
1	*B. bassiana* + *M. anisopliae*	1 ± 0.57a	1.3 ± 0.66a
2	*P. temperata* + *X. nematophila*	0.66 ± 0.66a	1 ± 0.57a
3	Control	18 ± 1.52b	7 ± 0.57b

Table II.- Number of holes/grain by new emerged F_1 *C. chinensis* in chickpea grains treated with different synergistic concentrations of *B. bassiana*+*M. anisopliae* and *P. temperates*+*X. nematophila*.

S No.	Concentrations 1×10^8 (conidia/ml)	No. of holes/ grain
1	*B. bassiana* + *M. anisopliae*	7.6 ± 0.33a
2	*P. temperata* + *X. nematophila*	5.6 ± 0.66a
3	Control	41.3 ± 0.33b

RESULTS AND DISCUSSION

Experiment No. 1

The lowest number of eggs (0.66) was counted in the synergistic dilution of *P. temperata*+*X. nematophila* as compared to synergistic dilution of *B. bassiana*+*M. anisopliae* (1) which is not significantly different with each other. Whereas the highest number of eggs (18±1.52) was observed in control where no synergistic dilution was used (Table I). Minimum number of holes were seen in synergistic dilution of *P. temperata* + *X. nematophila* and *B. bassiana* + *M. anisopliae* which is not significant with reference to each other. Maximum number of holes was recorded in control where no synergistic dilution was used (Table I). More number of holes F_1 emerged (7.6±0.33) in synergistic concentration of *B. bassiana*+*M. anisopliae* as compared to synergistic concentration of *P. temperata*+*X. nematophila* (5.6±0.66) (Table II). Inhibition rate of 81.34% of *C. chinensis* was observed using *B. bassiana* in combination with *M. anisopliae* whereas 86.40% was observed for combined use of *P. temperata* and *X. nematophila*. These results were significantly different with each other (Fig. 1).

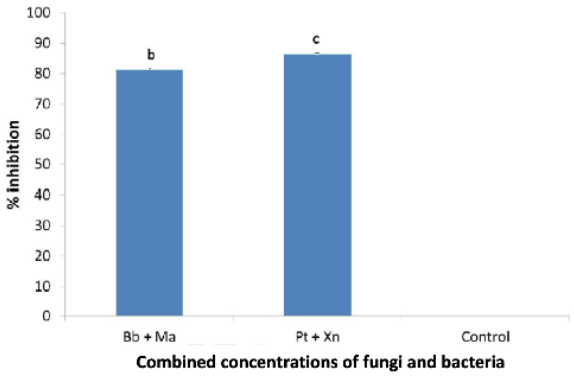

Fig. 1. Percent inhibition rate (Mean ± SEM) per grain laid by *C. chinensis* in chickpea grains treated with different synergistic concentrations of *B. bassiana* (Bb), *M. anisopliae* (Ma), *P. temperata* (Pt) and *X. nematophila* (Xn).

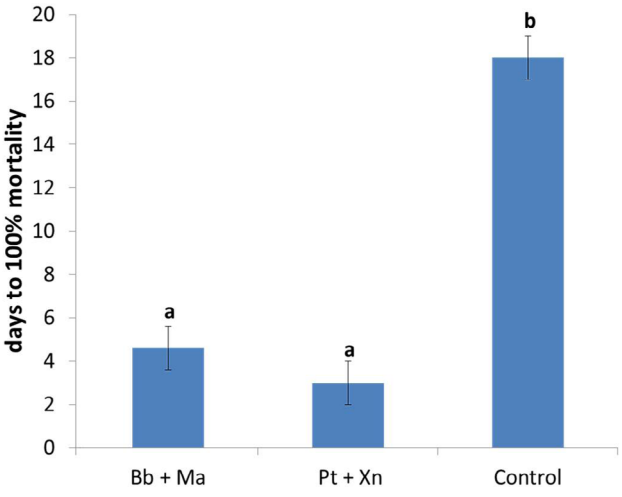

Fig. 2. Number of days till 100% mortality (Mean ± SE) per grain by *C. chinensis* in chickpea grains when treated with synergistic concentration of fungi (*B. bassiana* and *M. anisopliae*) and bacteria (*P. temperata* and *X. nematophila*)

More number of days (18) recorded to 100% mortality of F_1 in control as compared to individual treatments of synergistic concentrations of *B. bassiana*+ *M. anisopliae* (4.60) and EPB *P. temperata* and *X. nematophila* (3.0) which is significantly different (Fig. 2). The maximum weight loss (82.2%) was recorded in control as compared to individual synergistic concentration used. More weight

loss (5.2%) was measured in synergistic concentration of *B. bassiana+M. anisopliae* as compared to synergistic concentration of *P. temperata+X. nematophila* (2.2%) which is significantly different from each other (Fig. 3). Minimum damage (7.73%) is measured in synergistic concentration of *P. temperata*, *X. nematophila* as compared to synergistic concentration of *B. bassiana*, *M. anisopliae* (11.3%) which is significantly different from each other. Maximum damage (91.73%) was noted in control (Fig. 4).

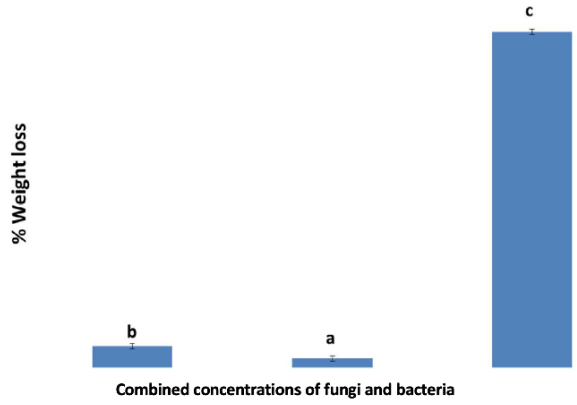

Fig. 3. Percent weight loss (Mean ± SEM) per grain laid by *C. chinensis* in chickpea grains treated with different synergistic concentrations of *B. bassiana+M. anisopliae* and *P. temperata+X. nematophila.*

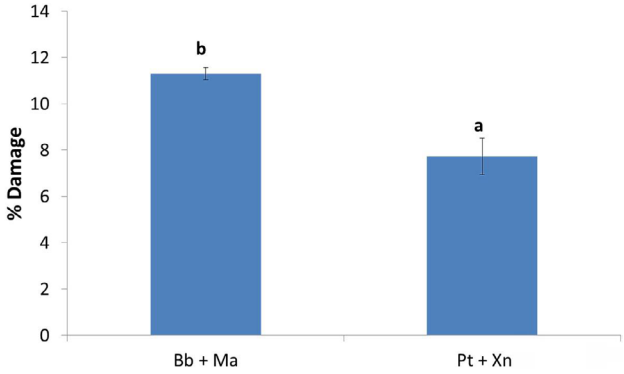

Fig. 4. Percent damage (Mean ± SEM) per grain laid by *C. chinensis* in chickpea grains treated with different synergistic concentrations of *B. bassiana+M. anisopliae* and *P. temperata+X. nematophila.*

Experiment No. 2

Mortality (%) of *C. chinensis* after 72 h was found absolute same as compared to 24 h and 48 h after application of synergistic dilutions of entomopathogenic fungi and bacteria.

Table IV.- Percent mortality (Mean±SE) of adults of *C. chinensis* when chickpea grains were treated with entomopathogenic *Beauveria bassiana* and DEBBM alone and in combination at different temperatures for 7, 14 and 21 days.

Treatments	Temperature		
	25°C	30°C	35°C
Exposed for 7 days			
D1+B1	21.67±1.88 bc	28.67±1.20cde	37.60±0.25abc
D1+B2	26.55±1.33 ab	34.67±0.88bcd	44.77±0.46 a
D1+B1	15.55±1.90 c	21.40±1.56 e	28.56±1.42 e
D2+B2	22.77±1.45 bc	27.35±2.50 de	29.95±1.50cde
D2+B3	25.45±1.20 ab	35.67±1.78 bc	37.28±1.68abc
DEB1+BB1	17.15±1.15 c	28.58±0.56de	23.48±0.58 e
DEB1+BB2	23.45±1.96abc	36.88±0.86bcd	30.67±1.22cde
DEB1+BB3	27.66± 1.40 ab	40.30±1.34 b	34.68±2.08bcd
DEB2+BB1	20.33±0.85 bc	30.52±2.05 cd	25.58±1.54 de
DEB2+BB2	25.10±1.20 ab	39.55±1.55 b	35.36±2.10abcd
D2+B2+BB3	31.67±1.67 a	50.40±2.20 a	43.55±1.90 ab
Exposed for 14 days			
DEB1	24.80±0.92 cd	41.87±0.96 de	52.96±0.33 bc
DEB2	32.58±1.48bcd	47.62±0.78 de	59.37±0.43 ab
BB1	22.85±1.70 d	35.45±1.51 e	35.52±0.49 c
BB2	30.72±1.46bcd	45.35±0.50 de	47.92±1.55 bc
BB3	37.25±1.28 ab	51.60±1.58 cd	55.29±1.62 b
DEB1+BB1	22.19±1.15 d	41.51±0.52 de	36.18±0.88 c
DEB1+BB2	31.44±1.96bcd	55.82±0.96bcd	49.60±1.20 bc
DEB1+BB3	41.67± 1.42 ab	62.38±1.64 bc	57.67±0.08 ab
DEB2+BB1	34.33±0.25 bc	52.22±1.07 cd	55.54±1.44 bc
DEB2+BB2	40.16±1.28 ab	67.50±1.53 ab	61.86±1.10 ab
DEB2+BB3	48.53±1.58 a	79.70±0.28 a	73.58±1.98 a
Exposed for 21 days			
DEB1	30.33±1.98bc	49.80±0.33 de	59.80±1.20abc
DEB2	43.50±1.48abc	66.69±0.68de	71.37±0.45 ab
BB1	28.45±1.75c	42.45±1.59 e	43.52±0.89 c
BB2	37.76±1.49abc	48.38±1.50 de	49.92±1.45 bc
BB3	45.23±1.68abc	57.63±1.08cd	61.29±1.68abc
DEB1+BB1	31.10±1.12 bc	56.24±0.51de	51.18±0.82 bc
DEB1+BB2	40.64±1.96abc	71.89±0.96bcd	60.60±1.28abc
DEB1+BB3	48.60± 1.43 ab	75.35±1.04 bc	67.67±0.78 ab
DEB2+BB1	36.35±0.25 bc	66.29±1.05 cd	61.54±1.42 abc
DEB2+BB2	47.18±1.20 ab	80.30±1.58 ab	74.86±1.17 ab
DEB2+BB3	55.55±1.32 a	90.76±0.56 a	85.58±1.66 a

Means followed by the same alphabets within the columns and rows are significantly similar ($P \leq 0.05$); Duncan, 1951 (DMRT). D1, 20g of DEBBM; D2, 40g of DEBBM ; B1, 6.69×105 (conidia/kg); B2 6.69×107 (conidia/kg); B3, 6.69×109 (conidia/kg).

Experiment No. 3

A large number of conidia were germinated when *B.*

bassiana was used with DEBBM at 30oC as compared to individual treatments at other temperatures.

It was observed that significant mortality of pulse beetle (90.76%) was recorded after 21 days, 14 days (79.70%) and after 07 days (50.40%) at 30oc temperature. when *B. bassiana* was used synergistically with DEBBM as compared to individual treatments(Table IV).

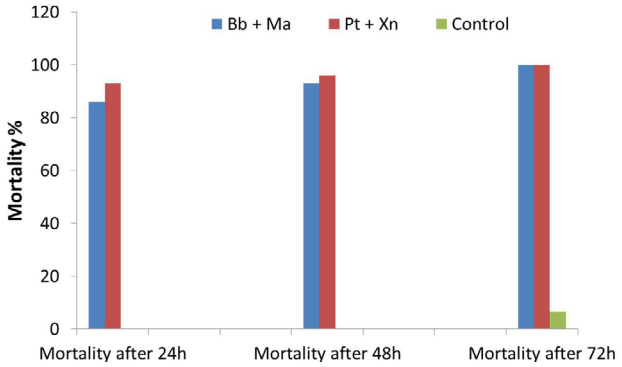

Fig. 5. Mortality (%) of *C. chinensis* after 24, 48 and 72 h after application of synergistic dilutions of entomopathogenic fungi and bacteria.

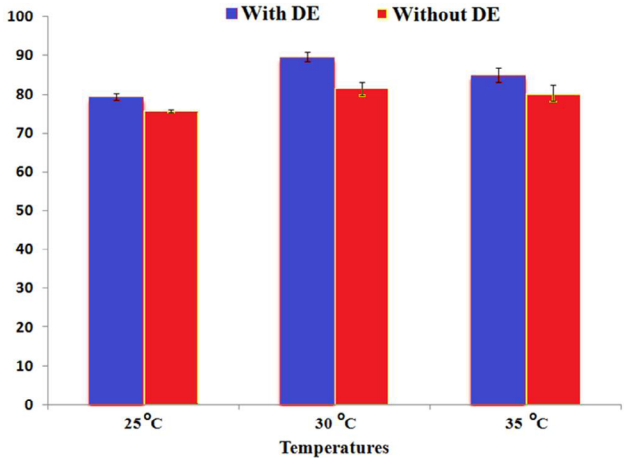

Fig. 6. Results of synergistic concentration of DEBBM with *B. bassiana.*

DISCUSSION

EPF and bacteria have been used very effectively against different insect pests both in field and storages. They have been used singly as well as in combination. Kryukov *et al*. (2009) were of the view that a synchronous coinfection of the Colorado potato beetle *Leptinotarsa decemlineata* (Say) with the EPF *Bacillus thuringiensis* spp. morrisoni Bonnifoi & de Barjak var. tenebrionis and hyphomycete *M. anisopliae* (Metsch.) Sorokin or *B. bassiana* (Bals.) Vuill leads to the rapid death of 95–100% of larvae. The synergistic effect of two pathogens is recorded at a relatively low hyphomycete titer (1–5×10^6 conidia/ml) and is evident in the mortality dynamics at all larval ages. These bacterial and fungal pathogens display no antagonism on artificial nutrient media. This microbial complex is highly efficient under natural conditions (80–90% larval mortality rate and no plant defoliation).

Similarly, Shaheen (2016) used EPF *Beauveria bassiana* as biological control agent to control pulse beetle *C. chinensis* in chickpea grains at different temperatures. Five concentrations (1×10^6, 1×10^7, 1×10^8, 1×10^9 and 1×10^{10} spores ml^{-1}) of commercially available conidia of *B. bassiana* were prepared. Mortality of pulse beetle was directly proportional to concentrations of *B. bassiana*. *B. bassiana* was less effective at 25ºC as compared to 30ºC. At 25ºC, highest mortality was recorded at concentration of 1×10^{10} spores ml^{-1} after 5, 10 and 15 days and vice versa. At 30ºC, *B. bassiana* showed better results and all pulse beetles died after 15 days at each concentration. This effective control strategy has significant contribution towards development of commercial microbial formulations of *B. bassiana* and is recommended to be a part of integrated pest management of pulse beetle.

According to Radha (2012), the efficacy of two EPF *M. anisopliae* (Deuteromycotina: Hyphomycetes) and *B. bassiana* (Ascomycota: Hypocreales) formulations were assessed against cowpea bruchid, *C. maculatus*. Five different concentrations of each formulation were used against each pest under investigation and compared with control insects. In liquid formulation of *B. bassiana* against *C. maculatus*, the percentage of adult mortality was 96 % in 5 x 10^6 conidial concentrations at 96 h interval and LT_{50} value was only 1.24%. Comparison of LC_{50}, LT_{50} values and mortalities indicated that in both assays, *B. bassiana* was consistently more virulent to bruchids than *M. anisopliae* because it had lower LC_{50} and LT_{50} and caused the highest mortality (96%) in treatment by suspensions containing 5×10^6 conidia/ml. *B. bassiana* had higher virulence than *M. anisopliae* against adult of cowpea weevil.

Sony and Kim (2010) described that two EPF, *P. temperata* subsp. *Temperata* (Ptt) and *X. nematophila* (Xn.) are symbiotically associated with the nematodes, *Heterorhabdis megidis* and *Steinernema carpocapsae*, respectively. There is little information on natural host ranges of the nematodes, but a significant difference in pathogenicity was observed between these two bacteria against the red flour beetle, *Tribolium castaneum*, in which *Photorhabdus temperata* exhibited more than six times higher pathogenicity than Xn. The pathogenic difference was not due to their inhibitory effect on phospholipase A_2 activity that is required for expression of immune response

of *T. castaneum*. The culture broths were fractionated into aqueous and organic extracts, most insecticidal activity remained in the aqueous extracts. The aqueous extracts of two bacteria contained proteins which showed different profiles.

Mburu *et al*. (2011) observed that isolates of the fungus *B. bassiana* have different levels of virulence and repellency against the termite *Macrotermes michaelseni*. They compared the volatile profiles and gene sequences of two isolates of the fungus with different levels of virulence and repellence to the termite. Subtractive bioassays showed that the repellency of each isolate was due to synergistic effects of a few constituents. As previously reported for isolates of *M. anisopliae*, some differences also were found in the nucleotide sequences of the two isolates of *B. bassiana*, suggesting a genetic basis for the observed intra-specific differences in their repellency and virulence against the termite.

The current findings of the study are in conformity with those of Correa *et al*. (2016). They reported that *P. luminescens* inhibited the growth of *B. bassiana* and *M. anisopliae* up to 40% by the secretion of secondary metabolites, whereas fungal extracts did not inhibit *P. luminescens*; this explains the in vivo interactions of these biological control agents. They established that on days 0, 2 and 4 there was an antagonistic interaction, while a synergistic interaction occurred on day 6. Therefore, the use of the interaction between *H. bacteriophora* HNI0100 with *M. anisopliae* Ma9236 and *B. bassiana* Bb9205 is an innovative alternative for the control of *P. xylostella*.

Similar results were observed by Jung and Kim (2006) when they revealed that *Xenorhabdus* sp. and *P. temperata* subsp. *temperata* (Ptt) are the symbiotic bacteria of the entomopathogenic nematodes, *Steinernema monticolum* and *Heterorhabditis megidis*, respectively. To increase their pathogenicity in the fifth instar, the bacteria should be delivered into the hemocoel. To this end, *Bacillus thuringiensis aizawai* (Bt) as a synergist was used to facilitate entry of the bacteria from the gut lumen into the hemocoel of *S. exigua* by its disruption of the insect gut epithelium. The bacterial mixture treatment was highly synergistic against the fifth-instar larvae of *S. exigua*. The synergistic effects were shown by the successful infection of *X.* sp. or *Ptt* in the insect haemocoel. This research shows a possibility that *Xenorhabdus* or *Photorhabdus* can be applied to kill *S. exigua* by oral treatment in a mixture with *Bt*.

Park *et al*. (2016) tested a hypothesis that bacterial immunosuppresants could enhance the susceptibility of mosquitoes (*Aedes albopictus* and *Culex pipiens* pallens) to *Bt*. *Bacillus thuringiensis* israelensis (BtI) was highly toxic to both culicid mosquitoes with median lethal concentration (LC_{50}, spores/ml) of 2.9105 and 2.2105 at 16 h after treatment, respectively. Addition of each bacteria-cultured broth enhanced BtI toxicity to these mosquito larvae. The LC_{50} values of BtI to *Ae. albopictus* larvae were reduced to 1.5105 in *Xn* mixture, 1.7105 in *Xh* mixture, and 1.9105 in *Ptt* mixture. The LC_{50} values of *Bacillus thuringensis* to *Cx. pipiens* pallens larvae were also reduced to 1.2105 in *Xn* mixture, 1.3105 in *Xh* mixture, and 1.5105 in *Ptt* mixture. Adding benzylideneacetone or oxindole produced from *Xn* and *Ptt* also enhanced *BtI* toxicities to these mosquito larvae. Based on these results, they developed a new mosquitocidal *Bt* formulation called "DipKill" consisting of 80% *Xn*-cultured broth, 10% *BtI* (1010 spores ml^{-1}), and 10% preservative. Dip-Kill at 1,000ppm was superior to a commercial *BtI* product at its recommended dose.

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Statement of conflict of interest

The authors declare no conflict of interest.

REFERENCES

Aslam, M., 2004. Pest status of stored chickpea beetle, *Callosobruchus chinensis* Linnaeus on chickpea. *J. Ent.,* **1**: 28-33. https://doi.org/10.3923/je.2004.28.33

Ahmed, Z., Aslam, M., Falak, N. and Illyas, M., 2011. Bio-efficacy of some plant extracts against chickpea beetle, *Callosobruchus chinensis* Linneaus (Bruchidae: Coleoptera) against chickpea. *Pakistan J. Zool*., **43**: 733-737.

Atif, H.M., Javed, N., Khan, S.A. and Ahmed, S., 2012. Virulence of *Xenorhabdus* and *Photorhabdus* bacteria and their toxins against juvenile's immobilization of *Meloidogyne incognita. Pak. J. Phytopathol*., **24**: 170-174.

Bakkali, F., Averbeck, S., Averbeck D. and Idaomar, M., 2008. Biological effects of essential oils-A review. *Fd. Chem. Toxicol*., **46**: 446-475. https://doi.org/10.1016/j.fct.2007.09.106

Correa, J.P., Sáenz-Aponte, A. and Rodríguez-Bocanegra, M.X., 2016. *In vitro* interaction of *Metarhizium anisopliae* Ma9236 and *Beauveria bassiana* Bb9205 with *Heterorhabditis bacteriophora*

HNI0100 for the control of *Plutella xylostella*. *SpringerPlus*, **5**: 2068.

Iqbal, M., Shaheen, F.A., Bhatti, A.R., Zia, A., Bodlah, I., Naz, F. and Fiaz, M., 2018. Effectiveness of *Photorhabdus temperata* and *Xenorhabdus nematophila* against *Callosobruchus chinensis* attacking stored chickpea grains. *Pak. Entomol*., **40**:95-103.

Jung, S. and Kim, Y., 2006. Synergistic effect of entomopathogenic bacteria (*Xenorhabdus* sp. and *Photorhabdus temperata* ssp. temperata) on the pathogenicity of *Bacillus thuringensis* ssp. aizawai against *Spodoptera exigua* (Lepidoptera: Noctuidae). *Environ. Ent.,* **35**: 1584-1589.

Khashaveh, A., Safaralizade, M.H. and Ghosta, Y., 2008. Pathogenicity of three Iranian isolates of the fungus, *Metarhizium anisopliae* (Metsch.) Sorokin (Deuteromycotina: Hyphomycetes) against granary weevil, *Sitophilus granarius* L. (Coleoptera: Curculionidae). *J. biol. Sci*., **8**: 804-808. https://doi.org/10.3923/jbs.2008.804.808

Kryukov, Y., Khodyrev, V.P., Yaroslavtseva, O.N., Kamenova, A.S., Duisembekov, B.A. and Glupov, V.V., 2009. Synergistic action of entomopathogenic hyphomycetes and the bacteria *Bacillus thuringiensis* ssp. morrisoni in the infection of Colorado potato beetle. *Appl. Biochem. Microbiol.,* **45**: 511-516. https://doi.org/10.1134/S000368380905010X

Mburu, D.M., Maniania, N.K., Hassanali, A. and Ndungu, M., 2011. Comparison of volatile blends and gene sequences of two isolates of *Metarhizium anisopliae* of different virulence and repellency toward the termite *Macrotermes michaelseni*. *J. exp. Biol.*, **214**: 956-962. https://doi.org/10.1242/jeb.050419

Nabaei, N.A., Mehrvar, S.M. and Bagheri, M., 2012. Efficacy of entomopathogenic fungi in combination with diatomaceous earth against *Callosobruchus maculatus* (Coleoptera: Bruchidae). *Acta Ent. Sin.*, **55**: 1282-1288.

Park, Y., Jung, J.K. and Kim, Y., 2016. A mixture of *Bacillus thuringiensis* subsp israelensis with *Xenorhabdus nematophila*-cultured broth enhances toxicity against mosquitoes *Aedes albopictus* and *Culex pipiens* pallens (Diptera: Culicidae). *J. econ. Ent.,* **109**: 1086-1093.

Radha, R., 2012. Insecticidal efficacy of entomopathogenic fungi in stored cowpea against cowpea bruchid, *Callosobruchus maculates* (F) (Coleoptera: Bruchidae). *Asian Acad. Res. J. Multidiscip.,* **1**: 117-130.

Rajendran, S. and Srinanjini, V., 2008. Plant products as fumigants for stored-product insect control. *J. Stored Prod. Res*., **44**: 126-135. https://doi.org/10.1016/j.jspr.2007.08.003

Shaheen, F.A., Akram, M.W., Rashid, M.A., Nadeem, M., Saeed, M., Husain, M. and Khalid, M., 2016. Biological control of pulse beetle *Callosobruchus chinensis* L. (Bruchidae: Coleoptera) in stored chickpea grains using entomopathogenic fungus *Beauveria bassiana* Balsamo. *J. Ent. Zool*., **4**: 1076-1083.

Shaheen, F.A., Akram, M.W., Rashid, M.A., Nadeem, M., Saeed, M., Husain, M. and Khalid, M., 2006. Resistance of chickpea (*Cicer arietinum* L.) cultivars against pulse beetle. *Pak. J. Bot*., **38**: 1237-1244.

Sony, S. and Kim, Y., 2010. Differential pathogenicity of two entomopathogenic bacteria, *Photorhabdus temperata* subsp. temperata and *Xenorhabdus nematophila* against the red flour beetle, *Tribolium castaneum*. *J. Asia-Pac. Ent.* **13**: 209-213.

Seogchae, J. and Yonggyun, K., 2006. Synergistic effect of entomopathogenic bacteria (*Xenorhabdus* sp. and *Photorhabdus temperata* ssp. temperata) on the pathogenicity of *Bacillus thuringiensis* ssp. aizawai against *Spodoptera exigua* (Lepidoptera: Noctuidae). *Environ. Ent*., **35**: 1584-1589. https://doi.org/10.1603/0046-225X(2006)35[1584:SEOEBX]2.0.CO;2

Tuan, A.N., Jeong, J.K., Seon, G.K. and Keun, K., 2009. Production of blastospore of entomopathogenic *Beauveria bassiana* in a submerged batch culture. *Mycobiology*, **37**: 218-224. https://doi.org/10.4489/MYCO.2009.37.3.218