ISJ 18: 108-118, 2021 ISSN 1824-307X

RESEARCH REPORT

Activity of detoxification enzymes in *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae) after exposure to *Beauveria bassiana* (Balsamo)

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 Accepted August 27, 2021

Abstract

Rhynchophorus ferrugineus is a devastating pest of palms worldwide. An integrated management strategy largely depends on chemical insecticides but due to concerns about human health risks and environmental pollution, it's essential to emphasize on the integrated pest management (IPM). In the current research the activities of detoxification enzymes esterases (EST), alkaline phosphatases (ALP), acid phosphatases (ACP), glutathione S-transferases (GST), and acetylcholinesterase (AChE) in *R. ferrugineus* collected from Punjab, Baluchistan, Sindh and Khyber Pakhtunkhwa (KPK) provinces of Pakistan were estimated after infection of *Beauveria bassiana* on 3rd-, 5th- and 7th-day post-treatment. The insects were exposed by immersion method with different concentrations of *B. bassiana*. The significant increase in activities of ALP (6.09), ACP (2.51), AChE (21.28) and EST (8.61) µmol/min/mg protein was observed in KPK population, while a significant increase in the activity of GST (5.23 µmol/min/mg protein) was recorded in Baluchistan population on 7th- day. The detection of elevated activities of detoxification enzymes showed the possibility of the resistance development against *B. bassiana* in *R. ferrugineus.*

Key Words: date palm; *Rhynchophorus ferrugineus*; entomopathogenic fungi; biocontrol; biochemical; detoxification enzymes; resistance mechanism

Introduction

Date palm *(Phoenix dactylifera)* is probably the oldest tree cultivated by humans and its production in Pakistan ranks at sixth position (Tavakolian *et al.,* 2013; FAO, 2014). Among notable insects damaging date palm, Red Palm Weevil, *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae) appears to be one of the cryptic insect (Molet *et al.,* 2011; Arab and El-Deeb, 2012). *R. ferrugineus* infestation was recorded in 50 % of date producing countries (Suma *et al.,* 2014; Wakil *et al.,* 2015). The native range of *R. ferrugineus* is Melanesia and South Asian countries and dispersal occurs due to transportation of ornamental palms across all continents (El-Mergawy and Al-Ajlan, 2011). *R. ferrugineus* prefers to attack young palm which are less than the age of 20 years because stem of young palm is juicy, soft, and easily penetrated by the insects. A single female of red palm weevil can give rise to more than approximately half billion of grubs in three generations. Moreover, *R. ferrugineus* is reflected as very disparaging insect of coconut palms (Ferry and Gomez, 2002). Despite huge efforts have been done to protect palm trees via synthetic chemicals, quarantine and other traditional methods (Abd-Elgawad, 1996), *R. ferrugineus* has proved to be stronger than these control measures and it has been entitled as the AIDS (acquired immune deficiency syndrome) of palm tree (Hanounik, 1998).

The growing demand of farmers to reduce chemical insecticides in agriculture, along with the environmental pollution and increased resistance to insecticides has provided huge impetus for the development of alternative control. An entomopathogenic fungus is alternative to the use of chemical insecticides (Sandhu *et al.,* 2012). Entomopathogenic fungi can penetrate the host cuticle and can be transmitted by contact with fungal spores or infected insects (Klein and Lacey, 1999), these are the main insect pathogens infecting beetles, because bacterial and viral diseases are rare among beetles (Hajek and St. Leger, 1994). The entomopathogenic fungi are usually host specific and are known to cause many physiological and biochemical changes in the host that alter the rate of growth, development and food utilization of

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Table 1 Median lethal time of *B. bassiana* virulence against *R. ferrugineus* at the highest tested concentration

Provinces	LT_{50} (Days)	(95 % FL)	Slope	χ^2	*Df*	*P*	N
Punjab	2.849	2.497-3.203	2.20 ± 0.22	6.472	6	0.372	80
Sindh	3.166	2.769-3.586	2.02 ± 0.22	1.314	6	0.970	80
Baluchistan	3.599	3.162-4.099	1.98 ± 0.22	3.972	6	0.680	80
KPK	3.027	2.753-3.300	3.17 ± 0.25	10.381	6	0.109	80

FL=Fiducial limits
P-values are based on Chi-square goodness of fit test.
N=Number of larvae used in the treatment including control.

the host (Butt *et al.,* 2016). *B. bassiana* is potential fungi against *R. ferrugineus* (Gindin *et al.,* 2006; Güerri-Agulló *et al.,* 2010; Ricaño *et al.,* 2013).

Insects routinely deal with many toxic substances that may be chemicals or microbial agents. Insects use enzymes including acetylcholinesterase (AChE), esterase (EST), alkaline phosphatases (ALP), acid phosphatases (ACP) and glutathione S-transferases (GST) as their defense mechanism to xenobiotic agents (Zibaee *et al.,* 2009a). Xenobiotic agents are compounds which might penetrate insect body and then enzymes enable insects to escape from these agents. The toxic chemicals are degraded by these enzymes without showing their action (Bogwitz *et al.,* 2005). The entomopathogenic fungi infected insects elevate the expression of GST, EST and AChE. The activation of detoxifying enzyme after fungal infection initiates its rapid degradation, catalyzation, hydroxylation and finally excretion (Wang *et al.,* 2004). The activities of EST and GST increased post treatment with entomopathogens in *Dendrolimus tabulaeformis* Tsai and Liu (Fan *et al.,* 2013) and elevated EST and GST in *Eurygaster integriceps* Puton were also detected post-treatment with *B. bassiana* (Zibaee *et al.,* 2009a). The AChE activity also increased in *Nilaparvata lugens* (Stål) after treatment with fungal metabolites and botanical insecticides (Nathan *et al.,* 2008).

For the management of *R. ferrugineus*, previous studies just focused on the use of insecticides. However, ecofriendly *B. bassiana* can challenge the voracious damage against *R. ferrugineus* (Qayyum *et al.,* 2020), but the role of detoxifying enzymes after its infection remains under explored. Thus, the present study was conducted to assess metabolic resistance development after treating *R. ferrugineus* with *B. bassiana*, as a baseline to suggest better management tactics.

Materials and methods

Insect collection and rearing

The adults and larvae of *R. ferrugineus* were collected from all four provinces of Pakistan i.e., Baluchistan, Punjab, Khyber Pakhtunkhwa (KPK) and Sindh. The insects were later on shifted to sterile cages (60×60×30cm) covered with muslin cloth. *Saccharum officinarum* was used as diet for adult *R. ferrugineus* that was refreshed after two days. The larvae were reared on artificial diet made by following the method described by Ahmed and Freed (2021a) which was refreshed after three days. The rearing conditions were maintained at 27 ± 2 °C temperature, 70 ± 5 % relative humidity and 12/12 hours L/D photoperiod.

Beauveria bassiana

The isolate of *B. bassiana* tested was Bb-01 and had been maintained in laboratory culture prior to the beginning of the study.

Fungal bioassay

3rd instar larvae of *R. ferrugineus* were subjected to bioassays. For this individual larva was dipped for 10-15s in concentrations of *B. bassiana* i.e., 3×10^8, 2×10^8, 1×10^8, 1×10^7 and 1×10^6 spores/mL. All concentrations were prepared in 0.1 % Tween 80 solution following the methodology of Alkhaibari *et al.* (2017). Eighty larvae were treated for each concentration and each concentration was replicated four time. While a total of 480 larvae were treated with different concentrations including a control which was treated with Tween 80 solution only. The larvae after treatment were shifted in Petri plates (2.5 cm diameter) with an artificial diet. The data on enzymatic activity was recorded on 3rd-, 5th- and 7th-days post treatment.

The pathogenicity of *B. bassiana* against KPK, Punjab, Sindh and Baluchistan were statistically non-similar (95 % FLs did not overlap) to each other. Nevertheless, lowest LC_{50} (1.3×10^7 spores/ml) was noted in the KPK samples, while samples from Punjab, Sindh and Baluchistan had LC_{50} values of 1.5×10^7, 5.3×10^7 and 1.02×10^8 spores/ml, respectively (Ahmed and Freed, 2021b).

Sample preparation for determining the enzyme activities

The samples (n= 4) were taken from the afore-mentioned assays to further assess the enzymatic levels in *B. bassiana*-treated *R. ferrugineus* on 3rd-, 5th- and 7th-days as described by Serebrov *et al.* (2006). Third instar larvae were crushed in 80 µL of

Table 2 Mean (± SE) enzyme activities in the *R. ferrugineus* after infection with *B. bassiana* across different concentration (Spores/mL), three post infection times and four different locations in Pakistan

	Beauveria bassiana				
Treatment	GST	AChE	ACP	ALP	EST
1×10^{6}	1.83 ± 0.08d	3.53 ± 0.39e	0.49 ± 0.02e	2.19 ± 0.09e	2.72 ± 0.21e
1×10^{7}	2.15 ± 0.11d	4.76 ± 0.57d	0.94 ± 0.03d	2.65 ± 0.12d	3.27 ± 0.23d
1×10^{8}	2.81 ± 0.14c	5.81 ± 0.68c	1.27 ± 0.04c	3.64 ± 0.11c	4.13 ± 0.27c
2×10^{8}	3.67 ± 0.15b	7.81 ± 1.05b	1.51 ± 0.03b	4.15 ± 0.14b	4.64 ± 0.32b
3×10^{8}	4.17 ± 0.17a	9.51 ± 1.34a	1.92 ± 0.04a	4.76 ± 0.14a	5.37 ± 0.33a
Control	1.36 ± 0.04e	1.45 ± 0.04f	0.34 ± 0.02f	1.53 ± 0.05f	1.41 ± 0.03f
Location					
Baluchistan	2.90 ± 0.23a	5.35 ± 0.76b	1.08 ± 0.07b	2.99 ± 0.15bc	3.41 ± 0.27bc
KPK	2.64 ± 0.21a	5.83 ± 0.73a	1.25 ± 0.09a	3.67 ± 0.21a	4.16 ± 0.29a
Punjab	2.21 ± 0.19b	5.22 ± 0.77b	0.95 ± 0.07c	2.74 ± 0.16c	3.23 ± 0.25c
Sindh	2.90 ± 0.22a	5.51 ± 0.73ab	1.03 ± 0.07bc	3.21 ± 0.17b	3.55 ± 0.26b
Day					
3rd day	2.26 ± 0.10c	2.34 ± 0.10c	0.98 ± 0.07c	2.83 ± 0.14b	2.48 ± 0.13c
5th day	2.62 ± 0.14b	3.05 ± 0.14b	1.07 ± 0.06b	3.21 ± 0.15a	2.97 ± 0.15b
7th day	3.11 ± 0.16a	11.05 ± 0.75a	1.19 ± 0.07a	3.43 ± 0.16a	5.31 ± 0.26a

Means with similar alphabets within columns, for each tested variable, are not significantly different (Tukey's HSD test, $p > 0.05$)

0.15 M NaCl with a mortar and pestle. The final volumes were adjusted to 900 µL per replication for centrifugation. The samples were spun at 10,000 rpm for 10 min, and supernatants were used to determine enzyme activities.

Protein determination

Protein contents in *B. bassiana*-treated larval samples of *R. ferrugineus* were measured by following the Bradford (1976) procedure.

Enzyme Assays

The activity of AChE was measured as explained by Ellman *et al.* (1961) using acetylcholine iodide (0.075 M) as a substrate. The samples were incubated in 0.1 mM of EDTA, 100 mM phosphate buffer (pH 7.2), 10 mM of 5,5′-dithiobis (2-nitrobenzoic acid), and 100 mM of acetyl-choline at 30 °C for 30 min. The variation in absorbance was recorded at λ of 412 nm for 4 min at 30 s interval. ALP and ACP activities were determined by following the method of Serebrov *et al.* (2006) with slight modification. The samples were mixed with 2.3×10^{-4} M *p*-nitrophenylphosphate in 0.05 Tris-HCl, pH, 8.8 for ALP, 0.05 M citrate phosphate buffer, pH, 5.0 for ACP and incubated for 2 h at 30 °C. 500 µL (0.05 M NaOH) was added for color development. The change in absorbance was noted at 410 nm for 4 min and 30 s intervals. GST activity was measured by using chloro-2, 4-dinitrobenzene1 mM with 5 mM reduced glutathione and 0.1 M Tris buffer pH 8.0 (Caballero *et al.,* 2008). The activity of the enzyme was evaluated by monitoring continuous changes in absorbance at 340 nm for 4 min at 25 °C. The extinction coefficient of CDNB (0.0096) was used to determine the total GST's activity (Rizvi *et al.,* 2018). EST activity was recorded by using 1 mM P-Nitrophenyl acetate and 50 mM phosphate buffer as substrate (Damayanthi and Karunaratne (2005).

In each replicate, 100 µL of 0.6 M aNa (or bNa) and 100 µL of phosphate buffer (pH 6.5) were added to 10 µL of *R. ferrugineus* homogenate. After 30 min incubation, 100 µL solution of Fast Garnett BC was mixed to stop the reaction. The changes were determined at λ of 405 nm as an end point calculated from standard curves of a- and b-Naphtol. Following Rizvi *et al.* (2018), extinction coefficient of *P*npa (176.47) was used to measure EST activities.

Statistical analysis

The mortality data of *B. bassiana* treated *R. ferrugineus* were examined by POLO Plus software which yielded LT_{50} values, 95 % confidence limits

Table 3 ANOVA results for release activities of detoxification enzyme in the *R. ferrugineus* after infection with *B. bassiana* across different concentration (Spores/mL), three post infection times and four different locations in Pakistan

Sources	*df*	Enzyme activity against *Beauveria bassiana*(µmol/min/mg)									
		AChE		GST		ACP		ALP		EST	
		F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Treatment (T)	5	614.2	<0.001	119.31	<0.001	394.87	<0.001	162.85	<0.001	206.92	<0.001
Location (L)	3	7.58	<0.001	15.85	<0.001	26.24	<0.001	24.69	<0.001	25.14	<0.001
Day (D)	2	3390.04	<0.001	36.1	<0.001	23.38	<0.001	19.56	<0.001	466.65	<0.001
T × L	15	0.68	0.7975^{NS}	1.73	0.051	1.25	0.2419^{NS}	1.92	0.0255	2.11	0.0123
T × D	10	286.22	<0.001	2.23	0.019	0.67	0.7503^{NS}	0.75	0.6778^{NS}	20.76	<0.001
L × D	6	4.65	<0.001	1.54	0.1693^{NS}	1.59	0.1534^{NS}	1.11	0.3579^{NS}	1.05	0.3941^{NS}
T × L × D	30	0.73	0.8376^{NS}	0.46	0.9929^{NS}	0.2	1.0000^{NS}	0.3	0.9999^{NS}	0.33	0.9996^{NS}
Error (*df*)	144										

^{NS} Labelled values are showing non-significant results ($p > 0.05$)

(FL), chi-square values and slope ± SE. Statistical analyses were undertaken with the linear model using a factorial analysis of variance (ANOVA) considering location, concentration effects and post-infection time and their interaction as factor against the dependent responses (i.e., enzyme activity). Further, concentration effects were compared across districts for each post-infection time. The significant ($p < 0.05$) means for above analyses were compared using Tukey's Honestly Significant Difference (HSD) multiple comparisons Test. Graphs were prepared by using Graph pad Prism, version 6.02.

Results

Median lethal time of B. bassiana *virulence against* R. ferrugineus

The infectivity of *B. bassiana* on *R. ferrugineus* and its LT_{50} values were calculated. The lowest LT_{50} value (2.849 days) was noted in Punjab population, while populations of KPK, Sindh and Baluchistan had values of 3.027, 3.166 and 3.599 days, correspondingly at highest concentration (Table 1).

Enzymatic response in R. ferrugineus *post infection with* B. bassiana

The results indicated the significant effects for concentration, location, and post-infection time towards AChE, GST and EST activities in *B. bassiana* treated *R. ferrugineus* (Table 2, 3). The activities of enzymes in *B. bassiana* treated *R. ferrugineus* increased in a highly concentration-dependent as well as time-dependent manner, i.e., enzyme activities increased after each concentration and post-infection time increase.

AChE, GST, EST, ACP and ALP activities were highest for KPK and lowest for Punjab populations. An effect for treatment × day was typically significant towards AChE, GST and EST activities. However, the location × day interaction was typically significant towards AChE activity.

AChE, GST, ACP, ALP and EST post infection responses to B. bassiana

In *B. bassiana* treated *R. ferrugineus*, the post-infection activities of AChE, GST, ACP, ALP and EST increased with increasing post-infection time. The releases were highest for seventh-days post-infection time and typically for the highest exposure concentration (i.e., 3×10^8 spores/mL) (Figure 1-5).

AChE

The KPK population of *R. ferrugineus* treated with *B. bassiana* showed the maximum AChE activities on the seventh-day i.e., 21.28 ± 0.78 µmol/min/mg protein *(*F = 186.78, df =23, *p < 0.001*) followed by Sindh, Baluchistan and Punjab populations with maximum AChE activities i.e., 20.95 ± 0.45, 20.61 ± 0.23 and 19.95 ± 0.34 µmol/min/mg protein, respectively, at the highest exposure concentration (Figure 1).

ACP

The KPK population of *R. ferrugineus* infected by *B. bassiana* showed the maximum activity of ACP on the 7th-day i.e., 2.51 ± 0.39 (F = 30.19, df =23, *p < 0.001*) at the highest concentration in 3×10^8 spores/mL followed 1.98 ± 0.03, 1.85 ± 0.08 and 1.86 ± 0.06 µmol/min/mg protein in Baluchistan, Punjab and Sindh, respectively (Figure 2).

ALP

The *B. bassiana* treatment on *R. ferrugineus* showed maximum activity of ALP on 7th-day in KPK population i.e., 6.09 ± 0.32 µmol/min/mg protein

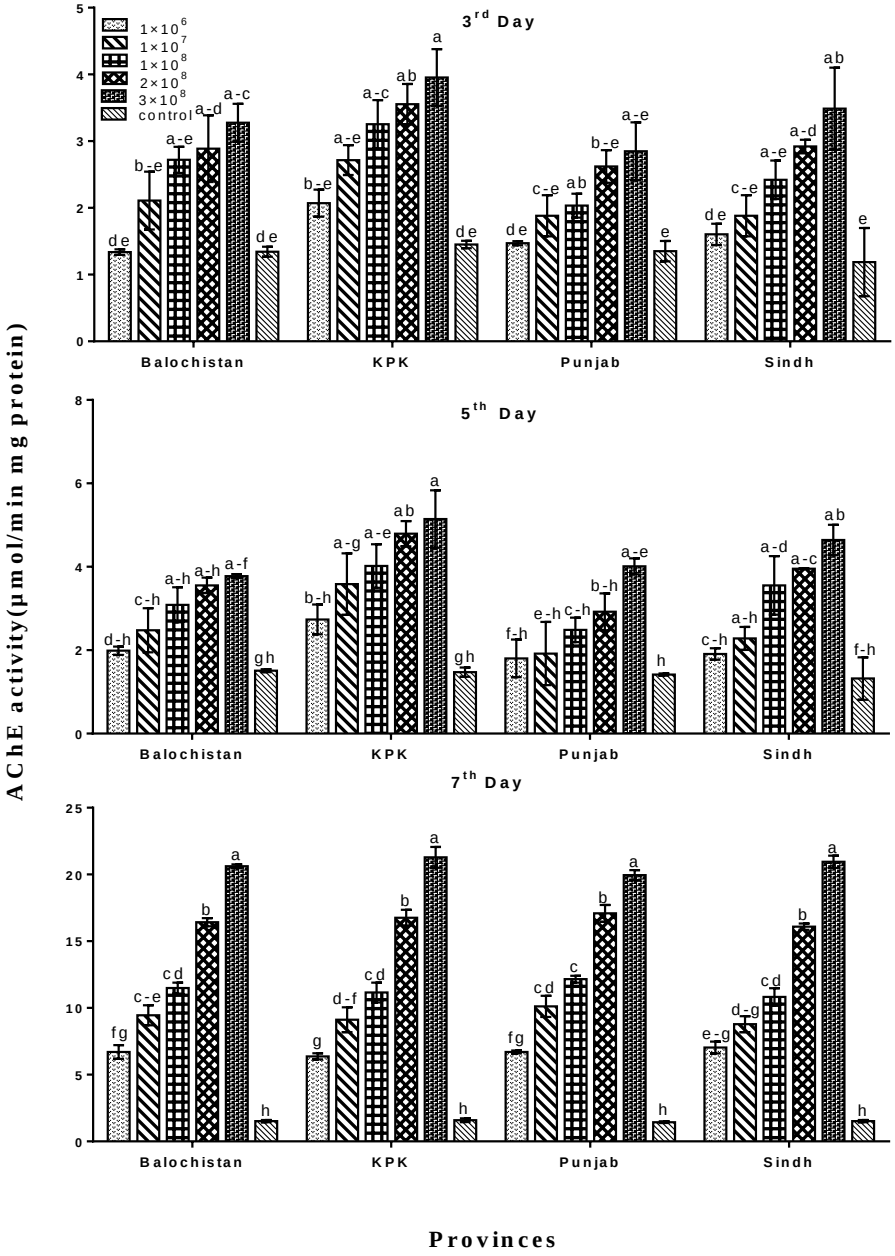

Fig. 1 Mean (± SE) activities of AChE in *B. bassiana* treated *R. ferrugineus* across three post-infections times for populations from different provinces of Pakistan. SE denotes standard error. Figure panels are showing post-ANOVA statistics for concentration effects according to 3rd, 5th and 7th day of treatment by location interaction. Bars within each panel labelled with similar letters are not significant from one another

(F = 12.78, df =23, *p* < *0.001*) at the highest exposure concentration followed by Sindh, Punjab and Baluchistan populations with maximum AChE activities i.e., 5.26 ± 0.32, 5.01 ± 0.51 and 4.51 ± 0.16, respectively (Figure 3).

EST

B. bassiana-treated *R. ferrugineus* showed a significant increase in EST activity. The maximum activity of EST was recorded in KPK population at the highest exposure concentration i.e., 8.61 ± 0.48 µmol/min/mg protein (F = 40.13, df =23, *p* < *0.001*) on 7th-day followed by 7.94 ± 0.52, 7.28 ± 0.54 and 7.27 ± 0.19 µmol/min/mg protein in Baluchistan, Sindh and Punjab, respectively (Figure 4).

GST

These results showed maximum GST activity in Baluchistan population 5.23 ± 0.38 µmol/min/mg protein (F = 11.77, df =5, *p* = *p* < *0.001*) on 7th-day at the highest exposure concentration followed by Sindh, KPK and Punjab populations with maximum

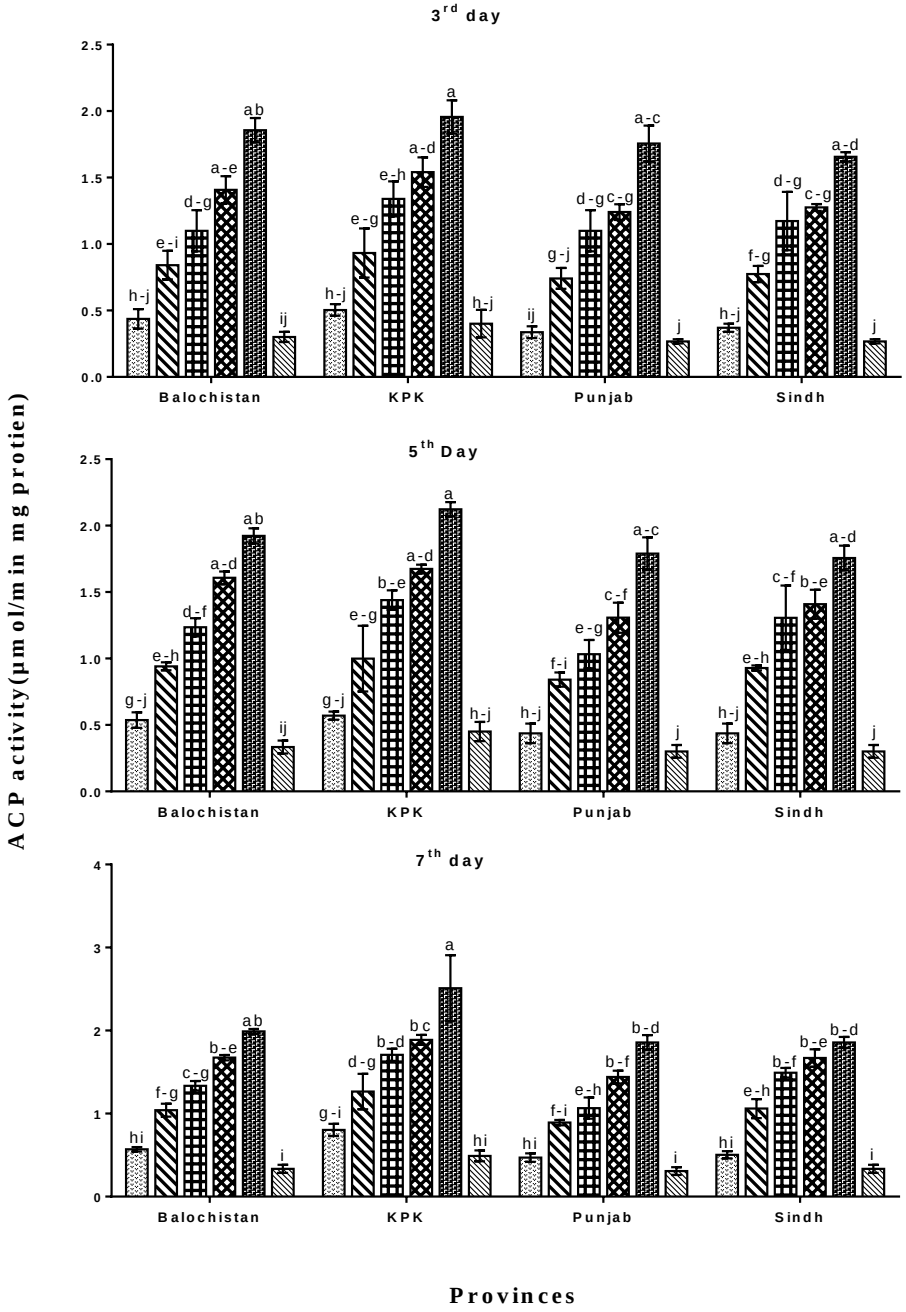

Fig. 2 Mean (± SE) activities of ACP in *B. bassiana* treated *R. ferrugineus* across three post-infections times for populations from different provinces of Pakistan. SE denotes standard error. Figure panels are showing post-ANOVA statistics for concentration effects according to 3rd, 5th and 7th day of treatment by location interaction. Bars within each panel labelled with similar letters are not significant from one another

AChE activities i.e., 5.17 ± 0.26, 4.87 ± 0.56 and 4.71 ± 0.51 µmol/min/mg protein, respectively (Figure 5).

Discussion

The enzymatic system is activated prior to infection by entomopathogens and maintains the regular physiological activities of an insect (Jun *et al.,* 2003). In the current study, treatment of larvae of *R. ferrugineus* with *B. bassiana* resulted in a significant increase in activities of the enzymes ALP, ACP, AChE, and EST in the KPK population only. In the Baluchistan population, only the activity of the GST enzyme was increased. The increased activities of detoxifying enzymes in insects against fungal infection may be due to activation of the immune response (Moorhouse *et al.,* 1993). The results of our research are consistent with those of Bilal *et al.* (2018) showing amplified GST and EST

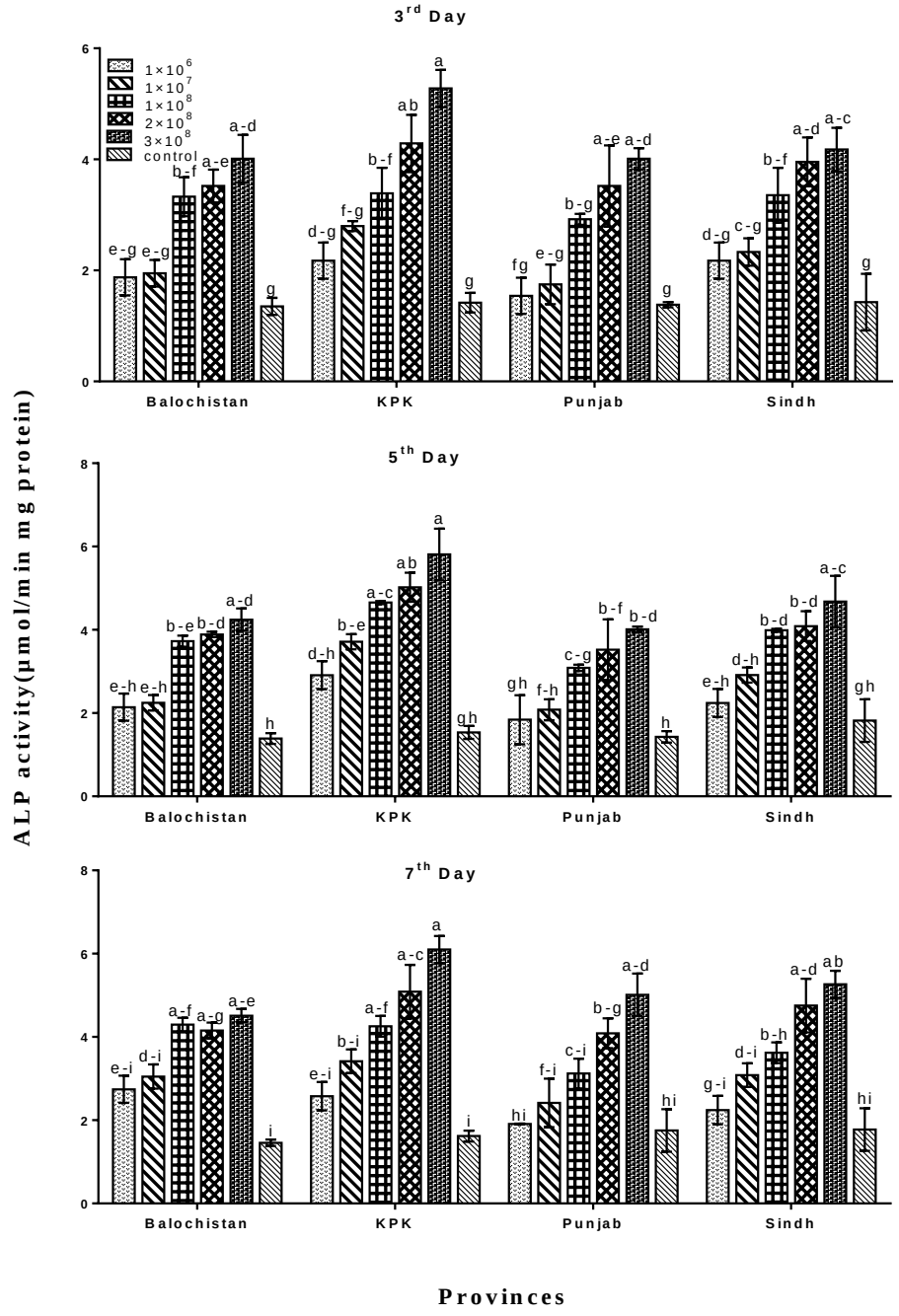

Fig. 3 Mean (± SE) activities of ALP in *B. bassiana* treated *R. ferrugineus* across three post-infections times for populations from different provinces of Pakistan. SE denotes standard error. Figure panels are showing post-ANOVA statistics for concentration effects according to 3rd, 5th and 7th day of treatment by location interaction. Bars within each panel labelled with similar letters are not significant from one another

levels in *Helicoverpa armigera* Hübner after *B. bassiana* infections. Similar results were reported by Serebrov *et al*. (2006) in *Galleria mellonella* L. in which the activity of GST and EST increased post fungal infection. Similarly, Naeem *et al*. (2020) reported increased activity of EST and GST in *Diaphorina citri* (Kuwayama) post fungal infection. The results of our research also relate to Farooq *et al.* (2018) who showed maximum GST activity in *Musca domestica* L. against the combined treatment of *B. bassiana* and imidacloprid.

Enzymes enable insects to escape from infection of microbial agents. The toxic chemicals are degraded by the detoxification enzyme prior to show their effectiveness (Bogwitz *et al.,* 2005). Our results showed that the application of different concentrations of *B. bassiana* to *R. ferrugineus* caused a significant increase in EST and GST

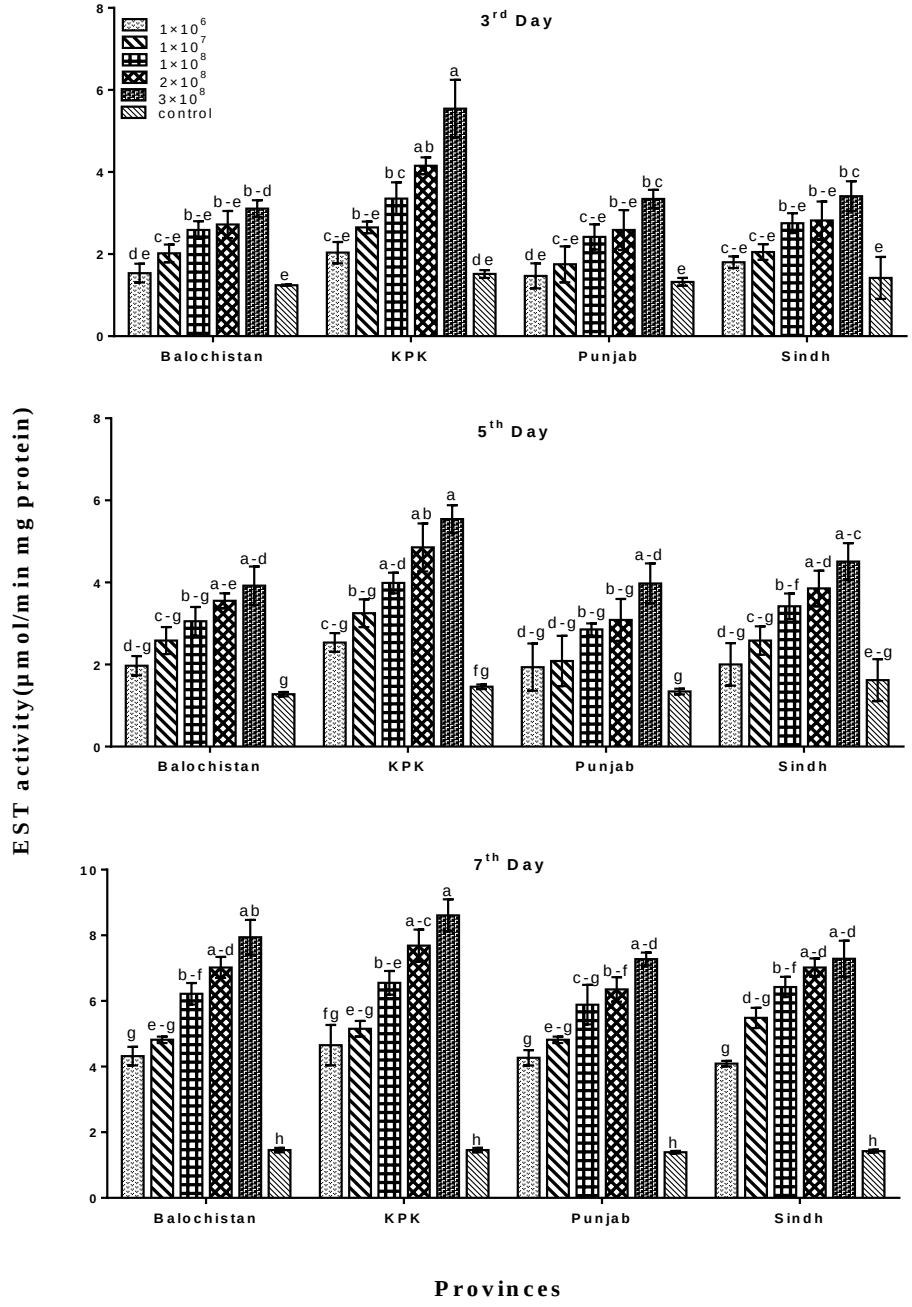

Fig. 4 Mean (± SE) activities of EST in *B. bassiana* treated *R. ferrugineus* across three post-infections times for populations from different provinces of Pakistan. SE denotes standard error. Figure panels are showing post-ANOVA statistics for concentration effects according to 3rd, 5th and 7th day of treatment by location interaction. Bars within each panel labelled with similar letters are not significant from one another

activities. Similar results were reported in *E. integriceps* which showed increased activities of EST and GST due to post-treatment with *B. bassiana* (Zibaee *et al.,* 2009a). Similarly, enhancement of GST and EST activities in locust was observed after fungal infections by Dubovskiy *et al.* (2012). In the current study, AChE activity increased after infection by *B. bassiana*. Our results are quite similar to the findings of Vidhya *et al.* (2016) who described elevated activity of AChE in *Spodoptera litura* (Fabricius) after the treatment of *B. bassiana.* The results of our study are also consistent with Bilal *et al*. (2018) who reported increased AChE activity in *H. armigera* post fungal infections. Contrary to this Cao et al. (2016) reported inhibiting activities of AChE in *Locusta migratoria* L. after fungal infection.

Insects use detoxification enzymes to show resistance against xenobiotics (Zibaee *et al.,* 2009b). Detoxification enzymes e.g., ALP and ACP

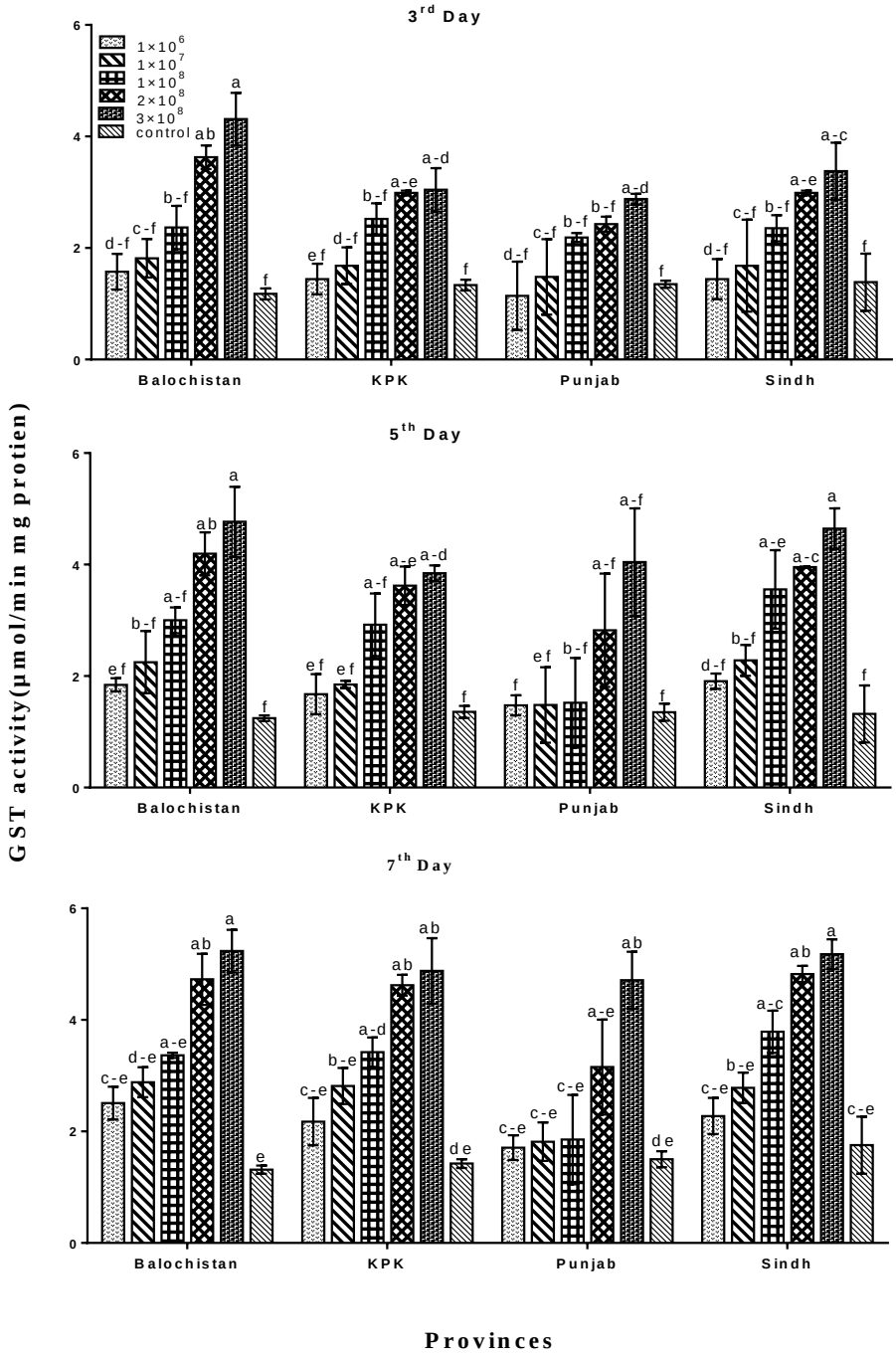

Fig. 5 Mean (± SE) activities of GST in *B. bassiana* treated *R. ferrugineus* across three post-infections times for populations from different provinces of Pakistan. SE denotes standard error. Figure panels are showing post-ANOVA statistics for concentration effects according to 3rd, 5th and 7th day of treatment by location interaction. Bars within each panel labelled with similar letters are not significant from one another

hydrolyze phosphomonoesters under alkaline and acidic conditions. In the current study, application of different concentrations of *B. bassiana* on *R. ferrugineus* showed increase in ALP and ACP activities. Similar enhanced expression of ALP and ACP as a defense mechanism was also reported by Bilal *et al.* (2017) in *H. armigera* after treatment with *B. bassiana.* Our results are also quite similar to the results of Vidhya *et al.* (2016) who showed an increased activity of ACP and ALP in *B. bassiana*-treated larvae of *S. litura.* Moreover, similar results were reported in *Schistocerca gregaria* post fungal infections (Xia *et al.,* 2000).

In conclusion, current study has described that *R. ferrugineus* infection with *B. bassiana* sharply increased detoxification enzyme activities mediating

detoxification and degradation of *B. bassiana*. This consequently increased the adaptation ability of insect body, particularly by decreasing their sensitivity to entomopathogenic fungi. This research provided novel options to develop very effective bio-control agents based on entomopathogenic fungi and their effect on *R. ferrugineus* due to the activities of enzymes.

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