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Metarhizium anisopliae and *Isaria fumosorosea* challenge the survival and immunity of the palm weevil, *Rhynchophorus ferrugineus* Olivier

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Abstract

Aim of study: Rhynchophorus ferrugineus Olivier is an invasive pest of palm worldwide. The use of insecticides by farmers for its management has been found insignificant. This study evaluated the potential use of entomopathogenic fungi for *R. ferrugineus* management with a particular focus on the fungal infection on the activities of different detoxification enzymes.

Area of study: Grubs and adults of *R. ferrugineus* were collected from various infested date palm fields in the four provinces of Pakistan.

Material and methods: Fungi *Isaria fumosorosea* (If-02) and *Metarhizium anisopliae* (Ma-M2) were evaluated against *R. ferrugineus*, and its immune responses were biochemically characterized.

Main results: The highest mortality rate was recorded at concentration 3×10^8 spores mL^{-1} on the 7th day post infection in the populations treated with *M. anisopliae* from Punjab, Khyber Pakhtunkhwa (KPK), Sindh and Baluchistan (93.75, 90.0, 90.0 and 81.25% respectively). *M. anisopliae* with lowest LC_{50} (1.1×10^6 spores mL^{-1}) from Sindh also proved to be the most lethal fungus against *R. ferrugineus.* Maximum acetylcholinesterase (AChE) and glutathione S-transferases (GSTs) activities were observed in Baluchistan (26.28 and 24.0 µmol min^{-1} mg^{-1} protein, respectively) and maximum esterases (EST) activity (35.4 µmol min^{-1} mg^{-1} protein) was observed in the KPK population on the 3rd-day post *I. fumosorosea* infection.

Research highlights: Fungal infection by *I. fumosorosea* caused a significant increase in AChE, GST and EST activities which may hinder *R. ferrugineus* development. However, *M. anisopliae*, to some extent, also inhibited enzyme activities and yielded a sudden increase in mortality. Future bio-pesticides could be developed for integrated pest management (IPM) of palm weevil.

Additional key words: biochemical resistance; bio-control; entomopathogenic fungi; integrated pest management; pathogenicity.

Abbreviations used: AChE (acetylcholinesterase); dpi (days post-infection); EST (esterases); FL (fiducial limit);GST (glutathione S-transferases); IPM (integrated pest management); KPK (Khyber Pakhtunkhwa, Pakistan); LCs (lethal concentrations).

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Introduction

The palm weevil, *Rhynchophorus ferrugineus* Olivier, is a destructive pest of palm trees, affecting approximately 29 different species of palm (Dembilio & Jacas, 2011; Hussain *et al*., 2013) including *Phoenix dactylifera* L. (date palm) (Giblin-Davis, 2001; Dembilio *et al*., 2009; Al-Dosary *et al.,* 2016). *R. ferrugineus* is present in almost 50% of *P. dactylifera* growing countries (Faleiro, 2006; Dembilio & Jaques, 2015) and causes severe production losses ranging from 0.7 to 10 tons ha^{-1} (Singh & Rethinam, 2005). *R. ferrugineus* is considered endemic to subcontinental countries; its first appearance in Pakistan was reported around the early 1980's which declined date production by 10–20% (Baloch *et al*., 1992) and by 30% in the Gulf region, while the Middle-East has lost US$ 103.66 million owing to injury caused by *R. ferrugineus* (El-Sabea *et al*., 2009). The geographic origin of *R. ferrugineus* is thought to be Melanesia and South East Asia (Ferry & Gomez, 2002) and dispersion has occurred due to the transportation of different palms around the globe (El-Mergawy & Al-Ajlan, 2011).

R. ferrugineus presents enormous management challenges due to its cryptic life cycle. Conventional methods for the control of *R. ferrugineus* consist of sanitary actions, use of pheromone traps, year-round surveillance, and the continued use of synthetic chemical insecticides which are applied by many methods, including axil filing, wound dressing, injection, spraying and fumigation (Cabello *et al*., 1997; Abraham *et al*., 1998; Al-Rajhy *et al*., 2005; Giblin-Davis *et al.,* 2013). Undoubtedly, the use of synthetic insecticides reduces *R. ferrugineus* infestations (Dembilio *et al*., 2010b). However, many factors, including environmental hazards, applicator safety, residue persistence and evolution of resistance have led experts to search for more environmentally friendly alternatives to control *R. ferrugineus* (Hussain *et al*., 2013). During the last decade, the development of sterile insect techniques and pheromone traps has shown some promise (Al-Ayedh & Rasool, 2010; Abdel-Moety *et al*., 2012). However, knowledge on the potential for microbiological control of *R. ferrugineus* remains scarce. Laboratory research on the infection of *R. ferrugineus* with various microorganisms, including bacteria (*Bacillus thuringiensis*) (Manachini *et al*., 2009), nematodes (*Steinernema affine*) (Llácer *et al*., 2010) and fungi (*Isaria fumosorosea, Metarhizium anisopliae* and *Beauveria bassiana*) (Ghazavi & Avand-Faghih, 2002; Shaju *et al*., 2003; Gindin *et al*., 2006; Dembilio *et al*., 2010a; Güerri-Agulló *et al*., 2010; Hussain *et al.,* 2013; Cito *et al*., 2014) has yielded diverse results in terms of insect mortality. Entomopathogenic fungi are an effective alternate to insecticides because they are cheap, environmentally friendly, and safe for non-target insects. With the ability to readily capture the host defense and degrade cuticle, high adoption rate, entomopathogenic fungi are cost-worthy choice (Hussain *et al*., 2014; 2015). The main benefit of using entomopathogenic fungi is their distinctive mode of action, which looks very promising as a low-impact alternative to chemical pesticides. Much effort has been invested in developing strains for the control of agricultural pests (Naeem *et al*., 2020). However, little is known about the potential for application of fungi to control *R. ferrugineus* in the field, as it passes almost all stages of life in the trunk of the tree, making the insect inaccessible for direct or contact treatment.

In response to infection with microbial agents or insecticides, many immune responses and/or metabolic processes can be triggered in an insect. Detoxifying enzymes are important modulators of the immune response of insects and refer to melanization and phagocytic ability (Gopalakrishnan *et al*., 2011). Enzymes activity can be stimulated by various xenobiotics and is associated with resistance to toxic chemicals (Müller *et al*., 2007). Increased enzyme activity may enhance the immune system ability to respond to xenobiotic challenges and may support healing (Cerenius & Söderhäll, 2004). Many mechanisms aid resistance to xenobiotics *i.e*., changed target site modification, increased excretion or decreased metabolic resistance and penetration mediated by detoxifying enzymes such as acetylcholinesterase (AChE), esterases (EST) and glutathione S-transferases (GST) help to develop resistance against xenobiotics (Kliot & Ghanim, 2012). These enzymes enable insects to escape xenobiotics by degrading the toxic chemicals before reaching target sites (Bogwitz *et al*., 2005). The increased activity of GST and EST produced via biotransformation of toxic chemicals is associated with increased metabolic detoxification of toxicant target sites, leading to development of resistance (Sokolova & Sundukov, 1999). Acetylcholinesterase plays a very important role in detoxification of xenobiotics by hydrolyzing the neurotransmitter acetylcholine at the cholinergic synapses, which leads to disruption of normal synaptic transmission (Kono & Tomita, 2006; Colovic *et al*., 2013). In general, the increased activity of these detoxifying enzymes reduces the insect's sensitivity to toxic chemicals (Serebrov *et al*., 2006). However, the functions of detoxifying enzymes in *R. ferrugineus* following fungal infection remain unexplored. Therefore, the current study was conducted to evaluate the effect of *I. fumosorosea* and *M. anisopliae* infections on the activities of detoxification enzymes, such as AChE, EST and GST, as related to pathogenicity to *R. ferrugineus*, in order to develop improved management strategies for this pest.

Material and methods

R. ferrugineus collection and rearing

Grubs and adults of *R. ferrugineus* were collected by hand from various infested date palm fields in four prov-

inces of Pakistan *i.e*., Khyber Pakhtunkhwa (KPK), Baluchistan, Punjab and Sindh. Palm weevil infestation was recognized by the symptoms of oozing frass and sap along the date palm trunk. The insects were placed in plastic cages (30×60×60 cm) covered with mesh screens and reared at 27 ± 2°C, relative humidity 70 ± 5% and L/D photoperiod 12/12 h. The adults were raised on fresh, clean and uninfected sugarcane stems (refreshed after 24 h), while the grubs were raised in plastic cups with Ahmed & Freed (2021a)′s artificial diet (45 g corn flour, 47 g wheat flour, 45 g yeast, 4 g ascorbic acid, 1.6 g sorbic acid, 2 g benzoic acid, two capsules of Pharmaton (1.55 g capsule^{-1}), 2 capsules Tetracycline (250 mg) and 37 g agar in 875 mL of distilled H_2O).

Insect pathogenic fungal isolates

Local isolates of insect pathogenic fungi *I. fumosorosea* (If-02) and *M. anisopliae* (Ma-M2) used for the experiments were identified and maintained in the Laboratory of Insect Microbiology, Department of Entomology, Bahauddin Zakariya University, Multan, Pakistan. The isolates If-02 and Ma-M2 were grown on potato dextrose agar (PDA) plates, placed at 27°C in dark at 75-85% relative humidity for 15 days, and after 15 days of fungal growth, the spores were stored at 5°C for further use against the insects. Different concentrations were prepared for the fungal bioassays by scrapping spores into Tween® 80 solution (0.05%); the spore concentrations were counted by haemocytometer and then the required concentrations were prepared by the serial dilution method.

Fungal bioassay

To check the efficacy of different fungal isolates, we followed the method of Ahmed & Freed (2021b): third-stage *R. ferrugineus* grubs were taken and immersed for 25 s in five different conidial concentrations of *M. anisopliae* and *I. fumosorosea* (1×10^6, 1×10^7, 1×10^8, 2×10^8 and 3×10^8 spores mL^{-1}) (n = 80 grubs for each concentration were exposed to four replicates). In the control treatment the grubs were immersed in 0.05% Tween® 80 aqueous solution. A total of 480 grubs were tested in each bioassay including the control. Thereafter, the treated grubs were carefully placed on dry tissue paper to absorb excess moisture; and then were placed in plastic Petri plates containing the artificial diet as described above. For sporulation and mycosis, the treated grubs were placed at >75% relative humidity. Mortality data were recorded on the 3rd, 5th, and 7th day of treatment. Live grubs were collected from all treatments with *M. anisopliae* and *I. fumosorosea* on the above days and placed in a freezer (-20°C) prior to biochemical assay. The assays were performed under the previously described laboratory conditions.

Enzyme assay and protein determination

The frozen grubs (n = 4) from fungal treatments and controls were crushed in ~ 80 µL of 0.15 M NaCl, in 2 mL Eppendorf tubes with a small pestle. After crushing, the final volume was adjusted to 700 µL per replicate for centrifugation as described by Caballero *et al*. (2008) with modifications. The homogenates were centrifuged at 10,000 rpm for 5 min and supernatants were used to determine AChE, GST and EST activities.

The protein contents of *R. ferrugineus* grub treated with *M. anisopliae* and *I. fumosorosea* were determined according to the procedure of Bradford (1976). AChE activity was measured according to Ellman *et al*. (1961) using the substrate acetylcholine iodide (0.075 M); changes in absorbance were recorded at λ=412 nm after 4 min for 30-s interval. GST activity was assessed following Caballero *et al*. (2008) using as substrate 1 mM 1-chloro-2, 4-dinitrobenzene (CDNB); changes were recorded at λ=340 nm after 4 min at 30-s intervals. EST activity was determined following the method of Damayanthi & Karunaratne (2005) using 1 mM 4-nitrophenyal acetate as substrate; changes were determined at λ=405 nm after 4 min 30-s intervals. The spectrophotometer used was UV3000 (O.R.I. Germany).

Statistical analyses

The mortality data of fungal-treated *R. ferrugineus* were analyzed by using the US. Environmental Protection Agency (EPA) Probit software 1.5 (EPA, 1992) to estimate lethal concentrations (LCs), their 95% fiducial limits (FLs), chi square values and slope (Finney, 1971). The probabilities were calculated by chi-square (χ^2) and degrees of freedom (*df*). We considered LC_{50} values significantly different if 95% FLs were non-overlapping (Litchfield & Wilcoxon, 1949).

Effect of fungal concentration, location and post-infection time on percent mortalities and release activities of enzymes (*i.e*., AChE, GST and EST) post-infection were assessed using linear mixed-effect models, with replications fitted as random effect, and concentration, location, post-infection time and their interactions as fixed effects. The effects were studied separately between *M. anisopliae* and *I. fumosorosea* isolates. The data were tested for normality and homoscedasticity using Kolmogorow-Smirnov and Levene's tests, respectively. The percent mortality data were arcsine-square root transformed to improve compliance with these assumptions. The assumptions were approximately satisfied for enzyme activities data, so no transformation was required. Significant (p<0.05) treatment means, for each concentration, location, and post-infection time were separated by multiple comparison test of Tukey's honest significant difference (HSD). As the interaction between concentration and post-infection

Table 1. Pathogenicity against *Rhynchophorus ferrugineus* of *Isaria fumosorosea* and *Metarhizium anisopliae* entomopathogenic fungi isolates obtained from different locations.

Fungi	Provinces	LC_{50} (spores mL^{-1})	95% FL	Slope	χ^2	*df*	p	N
M. anisopliae	Punjab	1.2×10^6	1.8×10^5-3.8×10^6	0.44±0.07	7.14	4	0.1289	480
	Baluchistan	2.8×10^6	1.1×10^5-1.1×10^7	0.28±0.07	4.86	4	0.3023	480
	Sindh	1.1×10^6	1.6×10^5-3.85×10^6	0.43±0.07	7.15	4	0.1281	480
	KPK	3.8×10^6	7.1×10^5-10.1×10^7	0.44±0.08	6.60	4	0.1585	480
I. fumosorosea	Punjab	2.3×10^6	1.2×10^5 -1.0×10^7	0.28±0.13	8.99	4	0.0613	480
	Baluchistan	3.9×10^6	2.1×10^5-1.4×10^7	0.27±0.07	6.12	4	0.1903	480
	Sindh	3.4×10^7	3.1×10^6-2.5×10^8	0.22±0.07	0.73	4	0.9478	480
	KPK	4.8×10^6	3.7×10^5-1.6×10^7	0.29±0.08	0.62	4	0.9608	480

FL = Fiducial limits. *p* = probability. N = number of *R. ferrugineus* exposed. *df* = degree of freedom.

time was typically significant at most events, therefore, the concentration effects on percent mortality and enzyme activities were further compared between three post-infection times, separately within districts, using repeated measures (RM) analysis of variance (ANOVA), followed by Tukey's honest significant difference (HSD) multiple comparison treatment for separating significant effects. As we were interested in comparing concentration effects between post-infection times rather than between locations, ANOVAs performed did not compare locations. All analyses were performed in SPSS software, vers. 21 (IBM Corporation, Chicago, IL, USA). All means and standard errors in text and figures were calculated with untransformed data. Graphs were prepared by using GraphPad Prism, vers. 6.02.

Results

Pathogenicity of *I. fumosorosea* and *M. anisopliae* against 3rd instar grubs of *R. ferrugineus*

The pathogenicity of *I. fumosorosea* and *M. anisopliae* or LC_{50} values against *R. ferrugineus* were not significantly different (95% FLs overlapped) among the tested populations. However, the lowest LC_{50} value (1.1×10^6 spores mL^{-1}) was recorded in the Sindh population treated with *M. anisopliae* which proved to be more potent given the higher LC_{50} values (3.4×10^7) for *R. ferrugineus* treated with *I. fumosorosea* on the 7th-day post-fungal treatment (Table 1).

Rhynchophorus ferrugineus post infection mortalities and enzyme activities

Mixed linear model analyses revealed significant effects for concentration, location, and post-infection time on post-infection mortalities and AChE, GST and EST activities in both *M. anisopliae*- and *I. fumosorosea*-treated *R. ferrugineus* (Tables 2, 3). The mortalities and activities of enzymes in *M. anisopliae*- or *I. fumosorosea*-treated *R. ferrugineus* increased in a highly concentration-dependent as well as time-dependent manner, *i.e.*, mortalities and enzyme activities increased with increasing concentration and time of infection. *Isaria fumosorosea*-treated *R. ferrugineus* mortality was greatest in populations from Punjab and KPK and lowest in populations from Sindh and Baluchistan. The Punjab and KPK populations showed the greatest EST activities, whereas, Baluchistan populations had the highest AChE and GST activities. The lowest activities of these three enzymes were recorded from Sindh, KPK and Punjab populations, respectively. *Metarhizium anisopliae*-treated *R. ferrugineus* showed greater mortalities for Punjab and KPK populations, and lowest for Baluchistan populations. The highest AChE, GST and EST activities were found in Punjab populations and the lowest in Baluchistan populations. Effects for treatment × day and treatment × day × location interactions were typically significant towards mortalities and enzyme activities; therefore we decided to investigate concentration effects according to post-infection time separately between districts.

Effects of post-infection time on *R. ferrugineus* mortalities

Treatment, sampling time and their interaction significantly affected mortality and AChE, GST, and EST enzyme activities (repeated measures ANOVAs; Tables S1, S2, S3 [suppl]). Table 4 shows that the mortality response of *I. fumosorosea* for 3×10^8 spores mL^{-1} (62.5 ± 0.87% to 78.75 ± 0.68%), 2×10^8 spores mL^{-1} (60.0 ± 0.52% to 71.25 ± 0.55%) and 1×10^8 spores mL^{-1} (58.5 ± 0.80% to 65.0 ± 0.50%), was similar between 5th and 7th-day, and these two were simi-

Table 2. Analysis (linear mixed effect model) results for percent mortalities and release activities of detoxification enzyme in the *Rhynchophorus ferrugineus* after infection with *I. fumosorosea* and *M. anisopliae* across three post-infection times and four different provinces of Pakistan.

	df	*F*	*p*	*F*	*p*	*F*	*p*
		% mortality					
		M. anisopliae		***I. fumosorosea***			
Treatment (T)	5	442.09	<0.001	670.22	<0.001		
Location (L)	3	31.48	<0.001	19.24	<0.001		
Day (D)	2	704.08	<0.001	710.98	<0.001		
T × L	15	2.20	0.007	1.64	0.064^{NS}		
T × D	10	24.88	<0.001	13.46	<0.001		
L × D	6	2.30	0.036	8.41	<0.001		
T × L × D	30	0.438	0.996^{NS}	1.92	0.004		
Error (*df*)	213	442.09	<0.001	670.22	<0.001		
		Enzyme activity against *M. anisopliae*					
		AChE		**EST**		**GST**	
Treatment (T)	5	601.64	<0.001	352	<0.001	239.7	<0.001
Location (L)	3	37.82	<0.001	32.9	<0.001	38.87	<0.001
Day (D)	2	81.33	<0.001	19.7	<0.001	62.15	<0.001
T × L	15	3.48	<0.001	3.07	<0.001	3.39	<0.001
T × D	10	9.83	<0.001	3.93	<0.001	3.3	0.001
L × D	6	2.89	0.011	9.22	<0.001	7.41	<0.001
T × L × D	30	1.75	0.016	2.64	<0.001	1.42	0.089^{NS}
Error (*df*)	142						
		Enzyme activity against *I. fumosorosea*					
		AChE		**EST**		**GST**	
Treatment (T)	5	1999	<0.001	2803	<0.001	1052.1	<0.001
Location (L)	3	70.97	<0.001	310.9	<0.001	225.42	<0.001
Day (D)	2	2862	<0.001	3117	<0.001	1268	<0.001
T × L	15	16.02	<0.001	55.8	<0.001	23.79	<0.001
T × D	10	477.6	<0.001	442.1	<0.001	279.09	<0.001
L × D	6	43.05	<0.001	60.17	<0.001	178.12	<0.001
T × L × D	30	11.88	<0.001	15.69	<0.001	20.06	<0.001
Error (*df*)	142						

^{NS} non-significant results (p>0.05). p = probability. *df* = degree of freedom AChE = acetylcholinesterase. EST = esterases. GST = glutathione S-transferases.

larly higher than on the 3rd day, suggesting that this fungus requires at least 5 days to cause maximum mortality. The mortalities by *M. anisopliae* were comparatively similar between tested concentrations up to the 5th day, but upon approaching the 7th day, mortalities significantly increased in 3×10^8 spores mL^{-1} (81.25 ± 0.27% to 95.00 ± 0.44%) and 2×10^8 spores mL^{-1} (71.25 ± 0.55% to 86.25 ± 0.27%) concentrations, meaning this fungus requires at least 7 days post inoculation to cause maximum mortality (Table 5).

AChE, EST and GST post infection responses to *I. fumosorosea*

In *I. fumosorosea*-treated *R. ferrugineus*, the post-infection activities of AChE, EST and GST decreased as time post-infection increased (Fig. 1). The release rates were highest at 3 days post-infection (dpi) and typically for the highest exposure concentration (3×10^8 spores mL^{-1}) that resulted in maximum AChE activities *i.e.*, 26.95 ± 0.15, 16.28 ± 0.52, 26.28 ± 0.52, 24.95 ± 0.15 µmol min^{-1} mg^{-1}

Table 3. Mean (±SE) percent mortalities and enzyme release activities in the *Rhynchophorus ferrugineus* after infection with *Isaria fumosorosea* and *Metarhizium anisopliae* across three post-infection times and four different provinces of Pakistan.

	I. fumosorosea (spores mL^{-1})				*M. anisopliae* (spores mL^{-1})			
	% Mortality	**AChE**	**EST**	**GST**	**% Mortality**	**AChE**	**EST**	**GST**
Treatment								
1×10^{6}	34.58±1.61 e	3.53±0.17 e	3.29±0.16 e	3.35±0.24 e	38.23±1.69 e	2.26±0.06 e	2.06±0.07 d	2.25±0.08 e
1×10^{7}	42.19±1.72 d	5.25±0.28 d	5.68±0.39 d	4.38±0.26 d	46.04±1.98 d	2.61±0.08 d	2.60±0.05 c	2.65±0.08 d
1×10^{8}	48.02±1.76 c	7.05± 0.51 c	9.76± 0.88 c	5.32± 0.46 c	52.71±2.12 c	3.08± 0.71 c	2.72± 0.05 b	3.16± 0.08 c
2×10^{8}	53.23±1.91 b	9.48± 0.83 b	14.24±1.26 b	7.19± 0.61 b	59.27±2.55 b	3.51± 0.06 b	3.04± 0.06 b	3.46± 0.09 b
3×10^{8}	58.13±2.13 a	13.16±1.38 a	16.93±1.62 a	9.40±0.98 a	66.28±2.97 a	3.69±0.05 a	3.43±0.09 a	3.70±0.11 a
Control	14.38 ±0.59 f	1.45 ±0.03 f	1.52 ±0.05 f	1.71 ±0.03 f	19.06±1.11 f	1.43 ±0.03 f	1.33 ±0.04 e	1.61 ±0.05 f
Location								
Punjab	44.24±2.24 a	6.97±0.86 b	9.67±1.23 a	4.23±0.40 c	50.42±2.57 a	2.99±0.12 a	2.69±0.11 a	3.16±0.14 a
KPK	43.06±2.31 a	6.02±0.55 c	9.51±1.24 a	4.48±0.42 c	46.04±2.53 b	2.77±0.12 b	2.33±0.09 b	2.82±0.11 b
Sindh	40.28±1.86 b	6.22±0.81 c	6.10±0.67 c	5.80±0.45 b	49.17±2.67 a	2.73±0.11 b	2.69±0.11 a	2.67±0.09 bc
Baluchistan	39.44±2.29 b	7.41±0.86 a	9.01±1.01 b	6.39±0.78 a	42.50±2.25 c	2.57±0.11 c	2.41±0.09 b	2.56±0.12 c
Day								
3 dpi	29.64±1.13 c	10.64±0.91 a	13.84±1.27 a	7.67±0.69 a	32.03±1.13 c	2.52±0.09 c	2.65±0.11 a	2.51±0.08 c
5 dpi	41.88±1.61 b	5.59±0.37 b	6.45±0.47 b	4.32±0.21 b	46.25±1.69 b	2.82±0.11 b	2.41±0.08 c	2.84±0.11 b
7 dpi	53.75±1.09 a	3.73±0.16 c	5.43±0.54 c	3.69±0.15 c	62.81±1.26 a	2.95±0.11 a	2.53±0.08 b	3.07±0.06 a

Means sharing similar letters within columns, for each tested variable, do not differ significantly (Tukey's HSD test, $p > 0.05$). dpi = days post-infection.

protein in Baluchistan, KPK, Punjab and Sindh, respectively (Fig. 1). The KPK population of *R. ferrugineus* treated with *I. fumosorosea* showed the maximum EST activities at 3 dpi (35.71±0.64 µmol min^{-1} mg^{-1} protein) followed by Baluchistan, Punjab and Sindh population with maximum EST activities (25.71 ± 0.56, 34.71 ± 0.46, 20.04 ± 0.99 µmol min^{-1} mg^{-1} protein, respectively), at the highest exposure concentration. The Baluchistan population of *R. ferrugineus* infected by *I. fumosorosea* showed the maximum activity of GST (24.14 ± 0.37 µmol min^{-1} mg^{-1} protein) at 3 dpi with the highest concentration of 3×10^{8} spores mL^{-1}, followed by the 5 dpi and 7 dpi (5.78 ± 0.01 and 5.81 ± 0.07 µmol min^{-1} mg^{-1} protein, respectively).

AChE, EST and GST post infection responses to *M. anisopliae*

Metarhizium anisopliae-treated *R. ferrugineus* populations showed lower AChE, EST and GST responses at 3 dpi than *I. fumosorosea*-infected *R. ferrugineus* populations. Unlike *I. fumosorosea*-infected *R. ferrugineus*, the release rates for three enzymes in *M. anisopliae*-treated *R. ferrugineus* populations were generally similar at 3 dpi for higher concentration (Fig. 2). The AChE releases rates were 3.76 ± 0.04, 3.82 ± 0.04, and 3.72 ± 0.16 µmol min^{-1} mg^{-1} protein in KPK, Baluchistan and Sindh, respectively, at 5 dpi with *M. anisopliae* and in Punjab, a non-significant difference of AChE activity was observed at 3 dpi, 5 dpi and 7 dpi. The maximum EST activity induced by *M. anisopliae* infection was detected in the Punjab population at 3 dpi (4.44 ± 0.24 µmol min^{-1} mg^{-1} protein) at highest concentration (3×10^{8} spores mL^{-1}) followed by Baluchistan and Sindh (3.44 ± 0.26 and 4.11 ± 0.28 µmol min^{-1} mg^{-1} protein respectively) at 3 dpi, and KPK (3.18 ± 0.04 µmol min^{-1} mg^{-1} protein) at 7 dpi with *M. anisopliae*. The Punjab population infected by *M. anisopliae* showed the maximum GST activity at 7 dpi (4.84 ± 0.05 µmol min^{-1} mg^{-1} protein) at the highest concentration (3×10^{8} spores mL^{-1}) followed by Baluchistan and KPK (4.51 ± 0.30 µmol min^{-1} mg^{-1} protein) at 7 dpi and Sindh (3.6714 ± 0.23 µmol min^{-1} mg^{-1} protein) at 5 dpi with *M. anisopliae*.

Discussion

Entomopathogenic fungi are potential bio-control agents for many species of insects including *R. ferrugi-*

Table 4. Mean (±SE) percent mortalities of *Rhynchophorus ferrugineus* from different locations after exposure of *Isaria fumosorosea* across three post-infection times.

Day	Concentration (spores mL^{-1})	Punjab	KPK	Sindh [a]	Baluchistan
3 dpi	1×10^{6}	23.75± 0.50 hi	22.50± 0.57 ij	23.75± 0.50 c	20.00± 0.81 hi
	1×10^{7}	32.50± 0.52 gh	30.00± 0.69 hi	31.25± 0.42 b	25.00± 0.74 gh
	1×10^{8}	38.75± 0.42 fg	35.00± 0.69 gh	35.00± 0.60 b	28.75± 0.80 f-h
	2×10^{8}	42.50± 0.43 e-g	37.50± 0.45 f-h	42.50± 0.43 a	32.50± 0.48 fg
	3×10^{8}	43.75±0.71 d-g	42.50±0.43 e-g	42.50±0.45 a	36.25±0.75 e-g
	Control	13.75 ±0.64 i	11.25±0.79 j	11.25±0.79 d	8.75 ±0.79 i
5 dpi	1×10^{6}	35.00± 0.69 g	35.00± 0.64 gh	32.50± 0.48 c	32.50±0.52 fg
	1×10^{7}	41.25± 0.71 e-g	42.50± 0.91 e-g	41.25± 0.39 b	38.75±0.39 d-f
	1×10^{8}	47.50± 0.43 d-f	50.00± 0.60 de	50.00± 0.60 a	45.00±0.60 c-e
	2×10^{8}	55.00± 0.55 b-d	55.00± 0.50 cd	52.50± 0.40 a	50.00±0.39 b-d
	3×10^{8}	68.75±0.59 a	67.50±0.34 ab	55.00± 0.55 a	52.50±0.40 bc
	Control	16.25 ±0.64 i	13.75 ±1.51 j	15.00±1.29 d	12.50 ±0.91 i
7 dpi	1×10^{6}	52.5± 0.40 c-e	47.50± 0.74 d-f	40.00± 0.60 b	50.00± 0.60 b-d
	1×10^{7}	62.5± 0.62 a-c	56.25± 0.78 b-d	52.50± 0.96 a	52.50± 0.70 bc
	1×10^{8}	65.0± 0.50 ab	65.00± 0.50 a-c	58.75± 0.64 a	58.50± 0.80 ab
	2×10^{8}	68.75± 0.86 a	71.25± 0.55 a	60.00± 0.52 a	71.25± 0.55 a
	3×10^{8}	72.50±0.80 a	75.00±0.84a	62.50±0.87 a	78.75±0.68 a
	Control	16.25 ±1.07 i	17.50 ±0.64 j	18.75 ±0.55 c	17.50±0.64 hi

Table is showing post-ANOVA statistics for concentration effects according to significant treatment by day interaction; see Table S1 [suppl]. [a] When the treatment by day interaction was non-significant, the pair-wise mean comparison between concentrations was performed within each of three post-infection times, separately. Means labelled with similar letters within columns do not differ significantly from one another (Tukey's HSD test, $p > 0.05$). SE denotes standard error. dpi = days post-infection.

neus (Cito *et al*., 2014). However, these fungi stimulate the immune system and alter the activity of detoxifying enzymes, so their effectiveness and modes of action against particular pest species need to be evaluated (Etebari *et al*., 2007; Lopes *et al*., 2011). Detoxification enzymes eliminate xenobiotics and maintain regular insect physiological processes (Nussenbaum & Lecuona, 2012). The role of detoxifying enzymes in induction of insect resistance using pathogens remains poorly explored. Hence, the aims of this study were to investigate the effect of *M. anisopliae* and *I. fumosorosea* on percent mortality and determine the role of detoxifying enzyme activities in the formation of insect resistance by the palm weevil, *R. ferrugineus*. We documented differences in mortality of *R. ferrugineus* with respect to regional populations, fungal conidial concentration and the role of detoxifying enzymes in survival of *R. ferrugineus.* The highest mortality (93.75%) was recorded in *M. anisopliae*-treated populations from Punjab, followed by populations from KPK, Sindh and Baluchistan (90.0%, 90.0% and 81.25% respectively), at the highest concentration (3×10^{8}) at 7 dpi. This is probably due to greater penetration of *M. anisopliae* spores into the host integument in the population from Punjab. Hyphae grow through the insect integument into the haemocoel, invade the tissues or organs, deplete nutrients and eventually cause death (Liu *et al*., 2011). The direct effect of physical damage caused by penetration of the hyphae into the insect integument is also an important aspect that requires additional research to develop measurements for the physical damage caused by the fungus itself.

Our results are also consistent with those of Fong *et al*. (2018), who reported *M. anisopliae* as the most pathogenic fungus, causing 100% grub mortality within 12 days of infection and a significant increase in mortality with an increase in conidial concentration. Similar results were reported by Francardi *et al*. (2012), who observed the highest efficacy of *M. anisopliae* against *R. ferrugineus* grubs and adults with 100% cumulative mortality. Other studies also suggest that *M. anisopliae* is the most potent fungus against *R. ferrugineus* (Gindin, 2006; Sewify & Fouad, 2006; Sabry *et al*., 2011; Kontodimas & Vassiliou, 2015; Abdel-Rahman & Abdel-Raheem, 2018; Yasin, 2019).

Preliminary studies have shown that an insect's enzymatic system is activated by entomopathogenic fungi in-

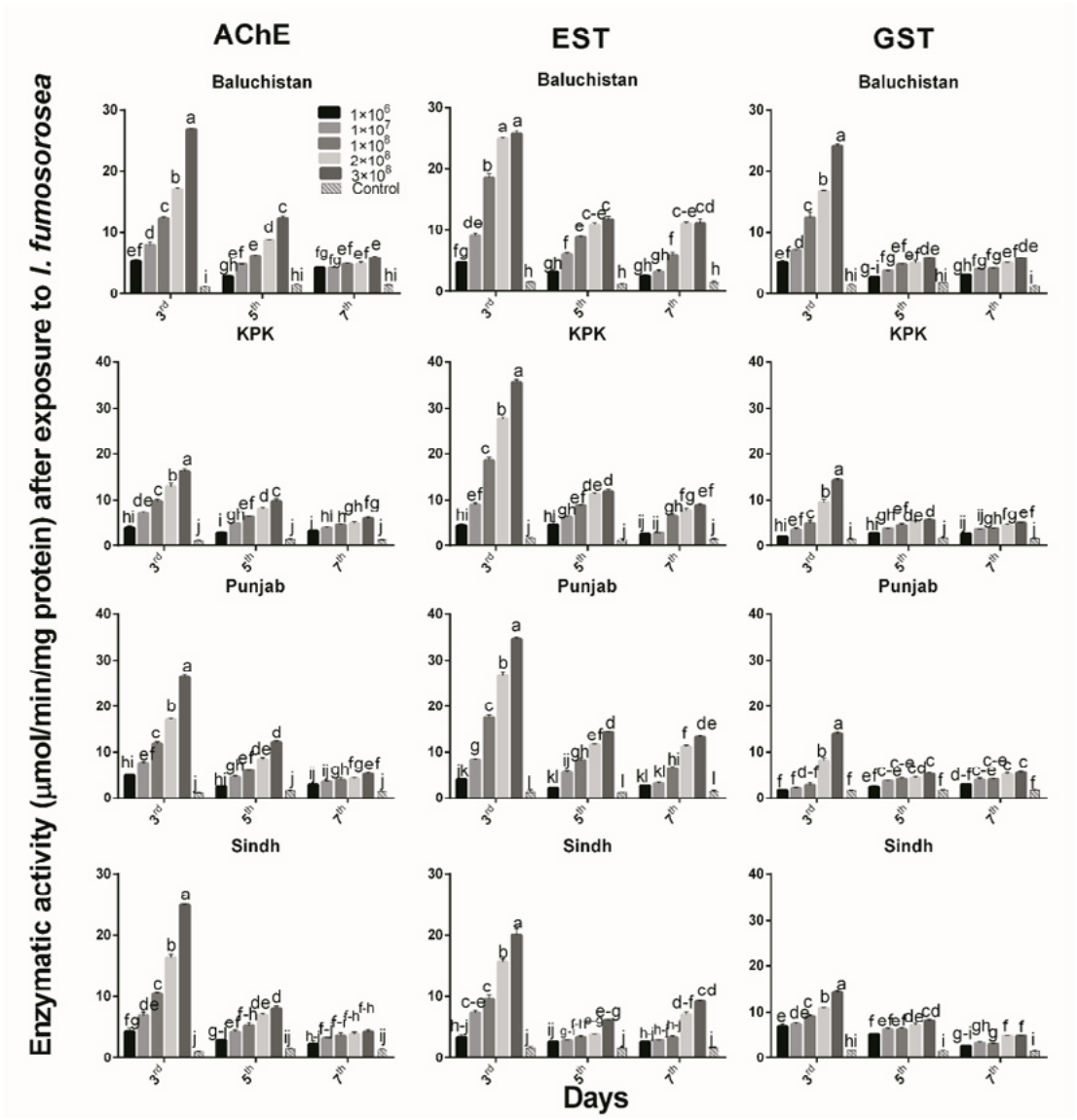

Figure 1. Mean (±SE) activities of enzymes (AChE, EST, GST) in *Isaria fumosorosea*-treated *Rhynchophorus ferrugineus* across three post-infections times for populations from different provinces of Pakistan. Figure panels are showing post-ANOVA statistics for concentration effects according to significant treatment by day interaction; see Table S3 [suppl]. SE denotes standard error. Bars within each panel labelled with similar letters do not differ significantly from one another, according to Tukey's HSD test, $p > 0.05$.

fection and this may enable the insect to maintain regular physiological functions (Song *et al.,* 2002; Jun *et al.,* 2003). In the present study, the activities of detoxification enzymes EST, GST and AChE were estimated 3, 5, and 7 days following fungal infection. The population of Baluchistan showed maximum activities of AChE and GSTs (26.28 and 24.0 µmol min^{-1} mg^{-1} protein, respectively), whereas, the maximum activity of EST in the KPK population (35.4 µmol min^{-1} mg^{-1} protein) was reached after 3 dpi with *I. fumosorosea*. These detoxifying enzymes contribute to defense reactions at the early stage of severe fungal infection (Dubovskiy *et al*., 2012). Comparable outcomes were previously documented with *Verticillium lecanii, M. anisopliae* and *B. bassiana* isolates (Kawachi *et al*., 2001). The results of our research are consistent with those of Vidhya *et al*. (2016) showing maximum GST activity in *Spodoptera litura* (Fabricius) after fungal infections. Similarly, Naeem *et al*. (2020) reported elevated activity of GST and EST in *Diaphorina citri* (Kuwayama) post fungal infection. Elevated activities of detoxifying enzymes in insects against fungal infection may result from activation of the immune system (Xu *et al*., 2017). Serebrov *et al*. (2006) reported elevated activities of GST and EST in *Galleria mellonella* L. post fungal infection. Moreover, Al-Jabr *et al.* (2017) reported elevated activities of EST and GST in *R. ferrugineus* post treatment with natural pesticides such as coumarin, methyl eugenol, diniconazole and rosmarinic acid.

Table 5. Mean (±SE) percent mortalities of *Rhynchophorus ferrugineus* from different locations after exposure of *Metarhizium anisopliae* across three post-infection times.

Day	Concentration (spores mL^{-1})	Punjab	KPK	Sindh	Baluchistan
3 dpi	1×10^{6}	27.50± 0.58 h-k	25.00± 0.82 h-j	26.25± 0.50 ij	25.00± 0.82 h-k
	1×10^{7}	35.00± 0.65 g-j	28.75± 0.46 h-j	33.75±0.81 hi	28.75±0.46 h-j
	1×10^{8}	41.25± 0.37 f-h	32.50± 1.02 g-i	40.00±0.61 gh	32.50± 1.02 g-i
	2×10^{8}	45.00± 0.61 fg	35.00± 0.69 f-i	45.00± 0.61 f-h	35.00± 0.69 f-h
	3×10^{8}	48.75±0.37 e-g	38.75±1.35 e-h	46.25±0.68 fg	38.75±1.35 e-h
	Control	18.75 ±2.86 k	13.75 ±1.51 j	13.75±1.51 k	13.75 ±1.51 k
5 dpi	1×10^{6}	37.50± 0.48 g-i	37.50± 0.45 e-h	35.00± 0.69 g-i	35.0±0.64 f-h
	1×10^{7}	45.00±0.57 fg	45.00± 0.57 d-g	46.25± 0.67 fg	41.25±0.39 e-g
	1×10^{8}	52.50±0.96 ef	52.50± 0.38 de	55.00± 0.55 d-f	47.50±0.43 d-f
	2×10^{8}	61.25±1.01 c-e	57.50± 0.38 cd	60.00.± 0.52 c-e	52.50±0.38 c-e
	3×10^{8}	73.75±0.28 bc	70.00±0.48 bc	70.00±0.50 bc	56.25±0.32 cd
	Control	22.50 ±2.47 jk	21.25 ±4.15 ij	20.00±2.23 jk	15.00 ±2.23 jk
7 dpi	1×10^{6}	55.00± 0.52 d-f	50.00± 0.77 d-f	52.50± 0.38 ef	52.50± 0.96 c-e
	1×10^{7}	67.50± 0.62 cd	60.00± 0.71 cd	65.00± 0.50 b-d	56.25± 1.06 cd
	1×10^{8}	73.75± 0.28 bc	68.75± 0.31 bc	73.75± 0.28 b	62.50± 0.36 bc
	2×10^{8}	83.75± 0.72 ab	78.75± 0.55 ab	86.25± 0.27 a	71.25± 0.55 ab
	3×10^{8}	93.75±0.50 a	90.00±0.43 a	95.00±0.44 a	81.25±0.27 a
	Control	25.00 ±0.91 i-k	23.75 ±1.67 h-j	21.25 ±0.55 jk	20.00±1.58 i-k

Table is showing post-ANOVA statistics for concentration effects according to significant treatment by day interaction; see Table S1 [suppl]. Means labelled with similar letters within columns do not differ significantly from one another (Tukey's HSD test, $p > 0.05$). SE denotes standard error. dpi = days post-infection.

Detoxifying enzymes make it possible for insects to avoid microbial agents by degrading xenobiotics before they reach target sites (Bogwitz *et al*., 2005). Our results showed that the use of different tested concentrations of *I. fumosorosea* isolate If-02 on *R. ferrugineus* increased EST, AChE and GST activities at 3 dpi. Similar results were reported by Dubovskiy *et al*. (2011) in *G. mellonella,* in which there was a significant increase in EST and GST activity after fungal infection. In our experiments, AChE activity increased after *I. fumosorosea* infection. Similarly, Bilal *et al*. (2017) reported elevated activity of AChE in *H. armigera* post-*I. fumosorosea* infections. According to Serebrov *et al*. (2006), *G. mellonella* caterpillars showed no significant increases in EST activity after *M. anisopliae* infection. Similarly, *R. ferrugineus* in our research showed no significant increase in EST activity after *M. anisopliae* infection. Similar results were obtained by Cao *et al*. (2016) showing inhibition of AChE and EST in *Locusta migratoria* L. after infection with *M. anisopliae*. Inhibited activities of detoxification enzymes showed that they play no significant role in the breakdown of the xenobiotics and may instead increase the susceptibility of insects to these toxic chemicals (Bakr *et al*., 2013).

The activity of detoxification enzymes in insects due to entomopathogenic fungi may vary both among and within species. Our study revealed differences in detoxification enzyme activities among *R. ferrugineus* populations from different provinces of Pakistan reared under the same laboratory conditions and subjected to the same entomopathogenic fungal treatments. Such differences may be related to adaptation to different environmental conditions in different palm growing regions or adaptive responses to prior exposure to fungal pathogens in some populations. The possible impacts of such population-level differences on development of effective pest management strategies should be explored through further research.

In conclusion, the present study demonstrated enhanced detoxification enzyme activities in *R. ferrugineus* after infection with two fungal species, *I. fumosorosea* and *M. anisopliae*. This suggests that *M. anisopliae* can be used for eco-friendly management due to its lower inductive effects on the inhibitory detoxifying enzyme

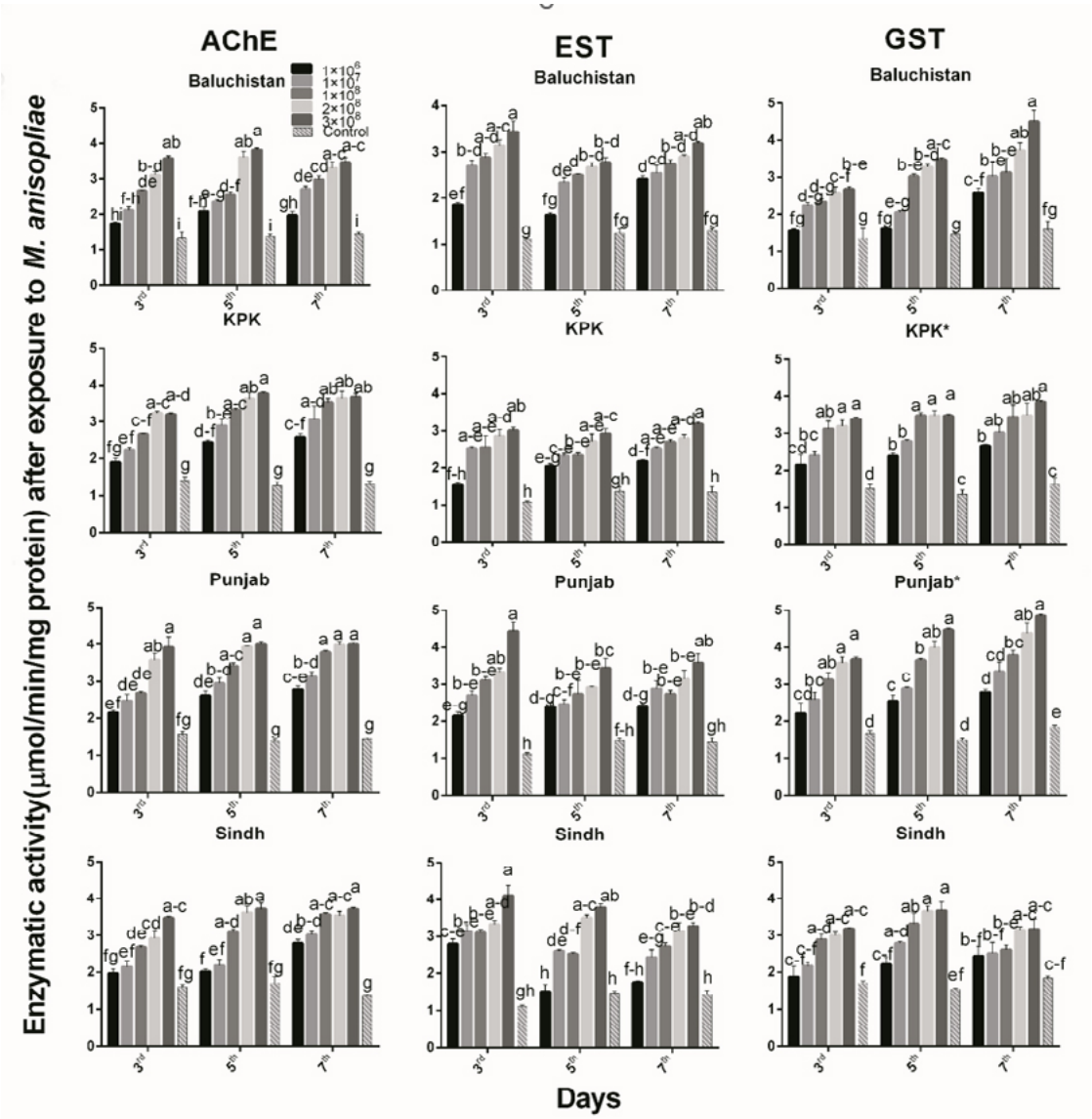

Figure 2. Mean (±SE) activity of enzymes (AChE, EST, GST) in *Metarhizium anisopliae*-treated *Rhynchophorus ferrugineu*s across three post-infection times for populations from different provinces of Pakistan. Figure panels are showing post-ANOVA statistics for concentration effects according to significant treatment by day interaction; see Table S2 [suppl]. SE denotes standard error. Bars within each panel labelled with similar letters do not differ significantly from one another, according to Tukey's HSD test, $p > 0.05$.

activities. This research opens up novel options for developing effective biological products based on entomopathogenic fungi in combination with enzyme inhibitors. More specifically, this may provide an effective pest management technique for the date palm industry through development of myco-insecticides targeting *R. ferrugineus*.

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Methodology: R. Ahmed.
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Writing – original draft: R. Ahmed, S. Freed.
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