Biochem. Cell. Arch. Vol. 18, No. 1, pp. 861-874, 2018 | www.connectjournals.com/bca | *ISSN 0972-5075*

PATHOGENICITY AND VIRULENCE OF THE ENTOMOPATHOGENIC FUNGI DEPEND ON SELECTIVE SUPPRESSION OF ANTI-OXIDATIVE AND DETOXIFICATION ENZYMES IN *TENEBRIO MOLITOR* (COLEOPTERA : TENEBRIONIDAE) LARVAE

Mandira Katuwal Bhattarai[1], Upendra Raj Bhattarai[1], Abolfazl Masoudi[1], Ji-nian Feng[1] and Dun Wang [1,2]

[1]Department of Entomology, Northwest A&F University, Yangling, Shaanxi, 712100, P. R. China.
[2]State Key Laboratory of Crop Stress Biology for Arid Areas, Northwest A&F University, Yangling, Shaanxi, 712100, P.R. China.
*e-mail: jinianf@nwsuaf.edu.cn, wanghande@nwsuaf.edu.cn, wanghande@yahoo.com

(Accepted 23 April 2018)

ABSTRACT : *Beauveria, Metarhizium* and *Isaria* are among the genera of well studied entomopathogenic fungi (EPFs) and has been widely formulated as mycoinsecticides. We quantified the virulence of six different isolates, two of each *Beauveria,Metarhizium* and *Isaria* spp. against 4th to 5th instar yellow mealworm, *Tenebrio molitor* larvae and described the early infection process and aspects of the host enzymatic immune response. We found that, Qin-21 (*B. bassiana*) and YYC-091 (*M. robertsii*) were among the most virulent isolates of all in order with LD_{50} of 9.12×10^4 and 3.4×10^5 conidia/g and Mean Survival Time (MST) of 3.44 ± 0.04 and 4.33 ±0.07 days at 2×10^8 conidia/g for respective treatments, whereas BSH-03 (*B. asiatica*) was the least virulent of all with LD_{50} of 4.1×10^7 conidia/g. Scanning electron microscopy revealed that Qin-21 and YYC-091 have comparatively faster germination rate within 12 h followed by vigorous extension of germ tube for later one within 24h. Furthermore, peroxidase (POD) and carboxylesterase (CarE) enzyme activity were inhibited throughout the infection period (POD, $P<0.01$) by the most virulent isolate Qin-21. Elevated catalase (CAT), superoxide dismutase (SOD), glutathione S-transferase (GST), alkaline phosphatase (AKP) for all the treatments implies the host's response for the oxidative stress because of infection. This research provides new insights into the pathogenicity and correlation of potential host physiological response to different fungal isolates with varying degree of virulence.

Key words : Entomopathogenic fungi, pathogenicity, *Tenebrio molitor*, enzyme activity, electron microscopy.

INTRODUCTION

Among the entomopathogens, fungi possess significant importance vis-a-vis bacteria and viruses for its ability to invade a host through direct penetration of the integument unlike needs to br orally ingested, making it more effective against sucking Hemipteran species (Payne, 1988; Carruthers *et al*, 1991) and non-feeding stages of the pests such as eggs and pupae (Charnley and Collins, 2007). In addition, they are also favored as an alternative to chemical pesticides because of their host specificity, minimal risks as well as newly discovered avenue of their symbiosis with plants promoting their growth and production (Behie *et al*, 2012; Sasan and Bidochka, 2012).

Among many genera of Entomopathogenic fungi (EPFs); *Beauveria*, *Metarhizium* and *Isaria* under the Phylum Ascomycota have been best characterized and widely studied (Borisade and Magan, 2014). These genera of EPFs possess cosmopolitan distribution and mostly been found in the terrestrial ecosystem such as in agriculture and forest landscapes, where soil is the main natural reservoir (Bidochka *et al*, 1998; Klingen and Haukeland, 2006; Sun and Liu, 2008). They have been evaluated for their pathogenicity, virulence, and efficiency for various types and stages of insect pests (do Nascimento Silva *et al*, 2018; Moura Mascarin *et al*, 2018; Negrete González *et al*, 2018). A number of them have also been commercialized by formulating as mycoinsecticides, such as Beauverin® from *Beauveria*, Biogreen® from *Metarhizium* and Priority® from *Isaria* to name a few of them (Copping, 2004; Kim *et al*, 2018), yet they cover only a small percentage of insecticide market (Khan *et al*, 2012). So, the improvements are demanding in every aspect, from isolation to development of new and more efficient EPFs for pest management.

Studies on EPF-host interaction has shed lights on

Corresponding author: WANG Dun, e-mail: wanghande@nwsuaf.edu.cn; wanghande@yahoo.com
Co-corresponding author: FENG Ji-nian, e-mail: jinianf@nwsuaf.edu.cn

the EPF's distinctive mechanisms to counter the host's defense system (Hajek and St. Leger, 1994; Vilcinskas and Götz, 1999; Dubovskiy *et al*, 2014; Pedrini, 2017). Improvement in EPF's innate abilities like adhesion, germination, penetration and growth has resulted in increased virulence (Robledo-Monterrubio *et al*, 2009) but they must disrupt or circumvent other crucial host's defense system, which includes, anti-oxidative and protective enzymes produced in response to the invading EPF and their toxins (Serebrov *et al*, 2006; Kamala Jayanthi *et al*, 2015). Nonspecific esterase like acetylcholinesterase (AChE), carboxylesterase (CarE) and glutathione S-transferase (GST) are most commonly involved in detoxifying enzymes in insects. These enzymes have been reported to be closely associated with the insect resistance and metabolism (Zhang *et al*, 2011b; Li *et al*, 2016). Besides, anti-oxidative enzymes like catalases (CAT), superoxide dismutase (SOD) and peroxidase (POD) are proven substantial to reduce the oxidative stress induced by EPF's toxins. But the differences of their individual response toward EPF of distant phylogeny and differing virulence has not been studied before.

In this study, we assessed the virulence and pathogenicity of six different isolates of entomopathogenic fungi from three genera (*Beauveria*, *Metarhizium* and *Isaria*) against *Tenebrio molitor* larvae. The early infection process was documented for the most virulent strains of all genera through scanning electron microscopy. Additionally, the physiological and biochemical changes in the larvae from 6 to 96-hour post infection (hpi) were quantified through the anti-oxidative and detoxification enzymes assays at different time points post infection.

MATERIALS AND METHODS

Insects

Commercially available *T. molitor* (yellow mealworm) larvae were reared on a clear plastic box (19×12×5 cm^3) with wheat bran substrate at 25±2°C, 55% RH, and a 16 h light and 8 h dark photoperiod. Healthy mealworms sized approximately 1.5 cm length (4^{th} to 5^{th} instar) were used for the subsequent experiments.

Fungal source, preparation of conidia and germination test

Six indigenous insect pathogenic fungal isolates within three genera namely, YYC-091 isolate (*M. robertsii*), SH-060 isolate (*M. pingshanse*), Qin-21 isolate (*B. bassiana*), BSH-03 isolate (*B. asiatica*), ILT-01 isolate (*I. cateniannulata*), ILDS-01 isolate (*I. cateniannulata*) were used in the study. These strains were isolated from different forest soils of China (Table1) between 2015 and 2017 and been characterized using morphological and molecular tools according to Rehner *et al* (2011) and Kepler *et al* (2014). Qin-21 is described by Masoudi *et al* (2017) and other's description is under manuscript preparation. However, morphological attributes of these fungi are revisited here. These isolates are now in the Lab of Insect Related Resources (LIRR) culture collection at Northwest A&F University, China.

In order to prepare the conidia for subsequent experiments, inoculants of each isolate stored in 20% glycerol at -80°C were sub-cultured on quarter strength, 4mm thick Sabouraud-dextrose-yeast extract agar medium (SDAY) composed of peptone 2.5 g/l, d-glucose 10 g/l, yeast extract 2.5 g/l, agar 20 g/l; in 90 mm Petri-dishes at 25 ± 2°C on constant dark for two weeks then were transferred to an incubator with 70% RH, 12:12 Light: Dark for one week before harvesting conidia with sterilized wooden spatula. Harvested conidia were collected in a sterile 50-ml Falcon conical tube (GLT™-Comb, Shanghai Kirgen Co., Ltd., Shanghai, China).

Germination test was also conducted for each isolate as described by Chen *et al* (2014). Harvested conidia were weighted and mixed with wheat bran as substrate. The conidial concentration was adjusted to 10^5 conidia/g after determining the concentration by diluting 1g of the mixture (conidia and substrate) with sterile water and counted in a hemocytometer (Neubauer-improved Hemocytometer, Lauda-Königshofen, Germany) using an inverted microscope (ECLIPSE TE2000-S, Nikon, Japan). The mixture was then incubated at 25 ± 2°C in a complete darkness. The moisture content of the substrate was adjusted to ~45% determining the difference between the wet and dry weight of the wheat bran substrate mixture. The cultivated mixture was sampled at 24 hours of incubation and serially diluted with double distilled water (ddH_2O) and counted in hemocytometer as mentioned above. Germinating conidial percentage was calculated per 200 conidia count per field of view using an inverted microscope (ECLIPSE TE2000 S, Nikon, Japan). The conidia with germ tube extended beyond its width were considered to be germinated (Inglis *et al*, 1997). Conidial viability >95% for all strains were assured before using in other experiments and remaining stock conidial powder were kept in a refrigerator at 4°C and used in next day right after evaluation of germination test (Safavi *et al*, 2007).

Virulence assay

Laboratory-based bioassay with four different conidial concentrations (2×10^8, 2×10^7, 2×10^6, 2×10^5 conidia/g) for each fungal isolate against *T. molitor* larvae was conducted for virulence assay. Series of conidial

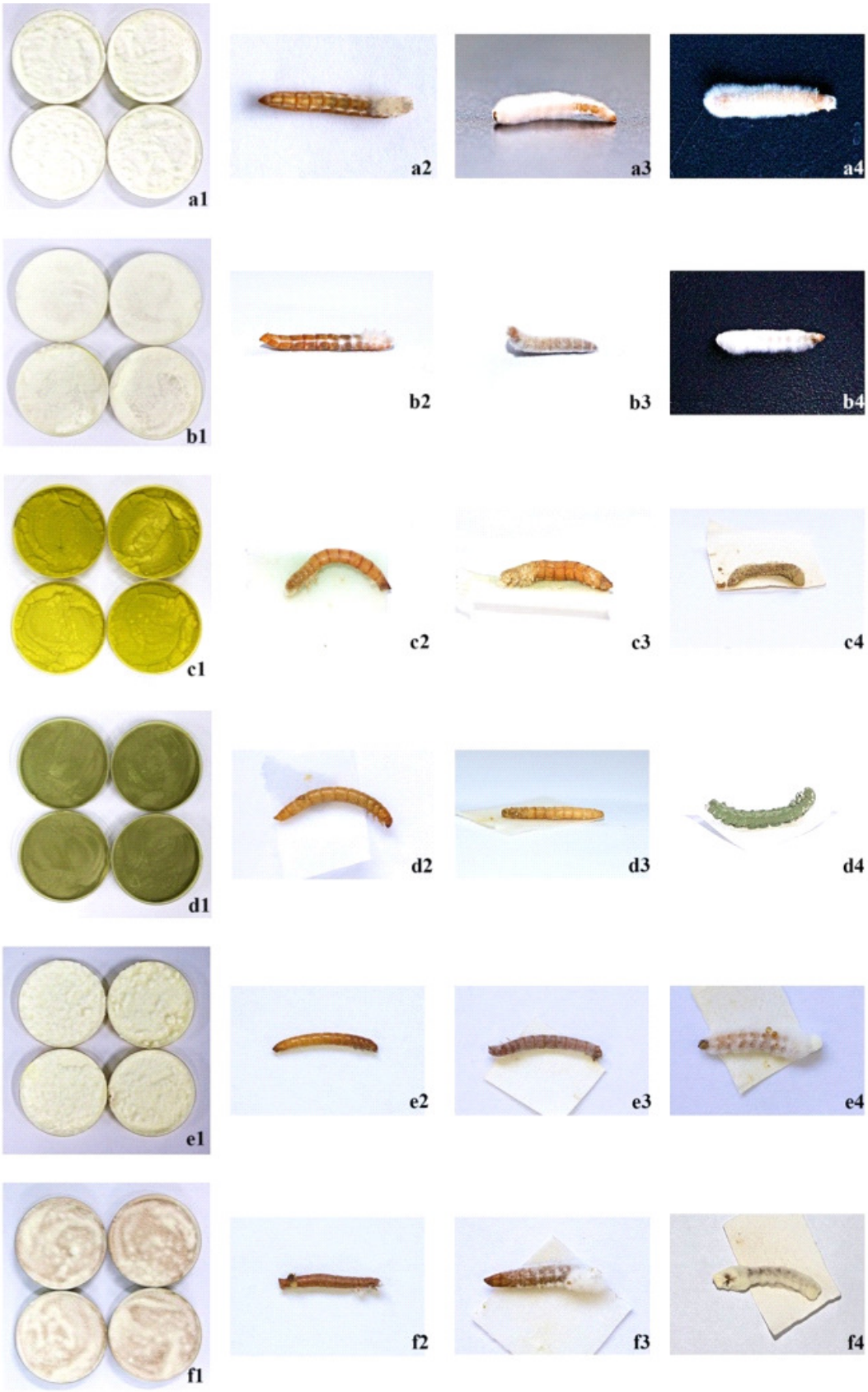

Fig. 1 : Appearance of fungal colony on SDAY medium and progressive symptoms of infection for all the tested isolates of EPFs. Colony of Qin-21 isolate (*B. bassiana*) showing white cottony appearance (a1) of BSH-03 (*B. asiatica*) (b1); light green colony of YYC-091 (*M. robertsii*) (c1); dark green colony of SH-060 (*M. pingshaense*) (d1); white colony of ILDS-01 (*I. cateniannulata*) (e1) and colony of ILT-01 (*I. cateniannulata*) (f1); hyphal extrusion and conidiation in series of progressive stages during infection of *T. molitor* larva by Qin-21 isolate (a1-a4); BSH-03 isolate (b2-b4); YYC-091 isolate (c2-c4); SH-060 isolate (d2-d4); ILDS-01 isolate (e2-e4); ILT-01 isolate (f2-f4).

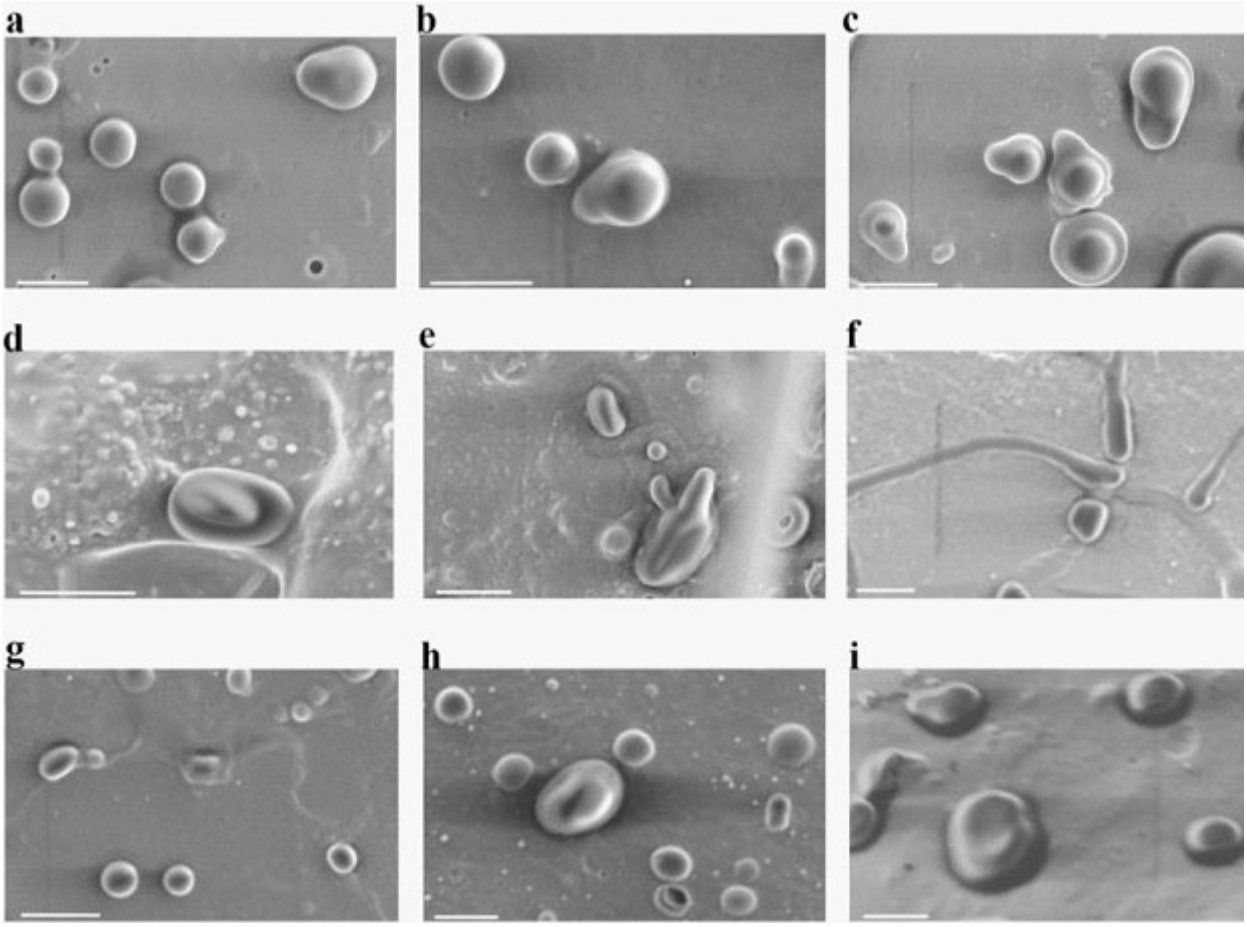

Fig. 2 : Scanning electron micrograph showing the early stage development of conidia on the surface of *T. molitor* larvae (6-24 hpi), illustrating the differences in germination efficiency of different isolates where YYC-091 isolate showed its dominance over others. Conidia showing round and globose shape, it's attachment and progression of germination of Qin-21 isolate (*B. bassiana*) at 6 hpi (a), at 12 hpi (b) and at 24hpi (c). oval to cylindrical shaped conidia of YYC-091isolate (*M. robertsii*) at 6hpi (d), its germination at 12hpi (e) and extension of germ tube on the surface of the larval epidermis at 24hpi (f). Round to elliptical shaped conidia of ILDS-01 isolate (*I.cateniannulata*) at 6 hpi (g) at 12 hpi (h) and at 24 hpi (i) on the surface of *T. molitor* larvae. White scale bar = 10 µm.

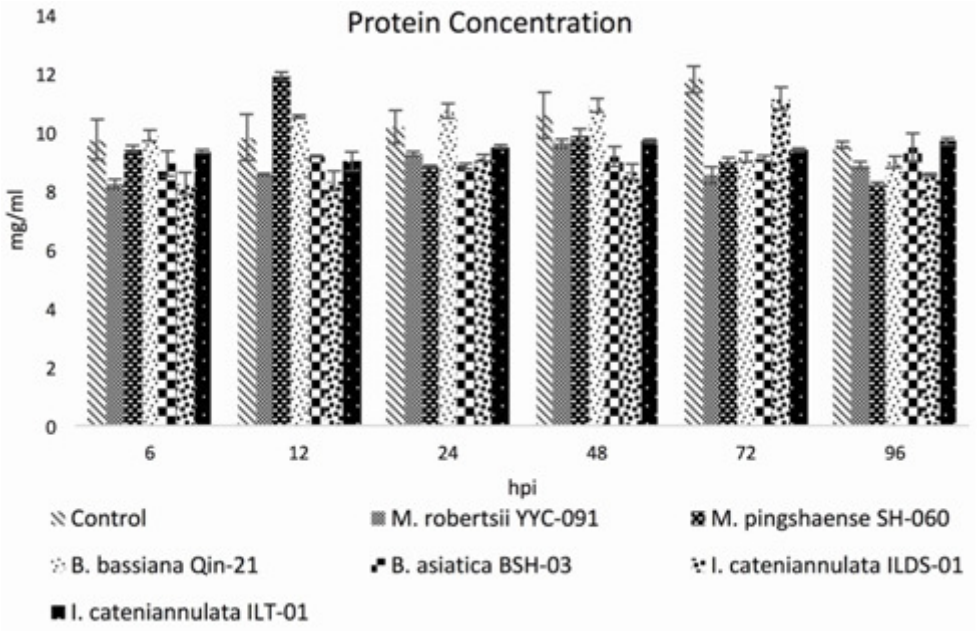

Fig. 3 : Change in the protein content in *T. molitor* larvae infected with different EPFs in series of time (6-96 hpi, X-axis). Data are expressed as mean ± SEM (n = 3).

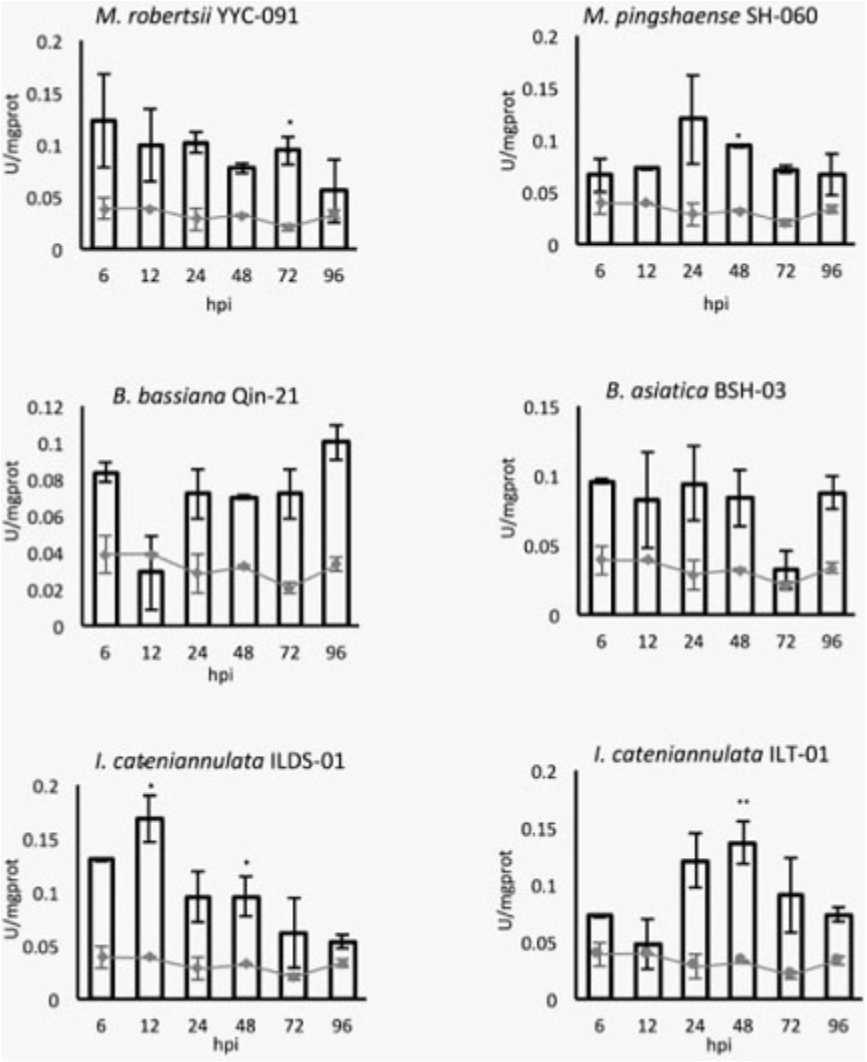

Fig. 4 : Comparative effect of different EPFs treatment over control on *T. molitor* larvae for Catalase (CAT) enzyme activity from 6 to 96hpi (X-axis). Enzyme activity on control group is presented by line graph and that of treatment by bar graph. Y-axis represents enzyme activity (U/mgprot). Data presents the Mean ± SEM of three replicates each with 5 larvae. Note: *, **, ***, ****, denotes significance at 5, 1, 0.1 and 0.01%, respectively (Dunnett's multiple comparisons test).

concentrations with sterile wheat bran as a substrate was prepared according to Chen *et al* (2014) and Masoudi *et al* (2017). Briefly, the conidial powder harvested and collected in falcon conical tube was weighted and mixed with sterilized wheat bran substrate; concentration was determined as mentioned above and the required concentration was prepared by adjusting the amount of conidia and substrate for each fungal isolate. 15 *T. molitor* larvae (4-5th instar) were placed along with 6 g of prepared mixture (substrate and conidia) inside a sterile 240 ml round glass tissue culture jar with plastic screw lids (Xuzhou Shengshi Glassware Co. Ltd. Jiangsu, China). Control treatment consisted of larvae with similarly prepared glass jar including substrate except for the conidia. All the jars were placed in an environmental chamber with 25 ± 2°C, 55 ± 7% RH and 12: 12 L: D photoperiod. Insect mortality was recorded daily till 18-days post infection. Dead insects were removed on the same day, surface sterilized with ddH_2O to remove attached substrate particles and placed individually on 24-well plastic boxes (12×6×2.5 cm^3) containing moist sterilized filter paper and then incubated on similar conditioned environmental chamber as mentioned above to encourage the fungal outgrowth. Death from fungal infection was confirmed by screening for the visible mycosis on the surface of the dead insects. The experiment was conducted with three biological replicates with 45 insects on each replicate per treatment and organized in a complete randomized design. Lethal dose (LD) was determined through Probit analysis (Finney, 1971) in SPSS Statistics Ver. 22.0 (Nie *et al*, 1970). Statistical significance difference (P<0.05) among the isolates for each concentration was calculated using analysis of variance (ANOVA), followed by a post hoc Tukey-Kramer test in SAS JMP statistical program version 13.2.0 (SAS Institute Inc., Cary, NC, USA). Mean survival time was calculated using OASIS 2 online tool (Han *et al*, 2016).

Preparation of tissue samples for Scanning Electron Microscopic examination

To observe the conidial shape, attachment and germination; single virulent strain: Qin-21 (*B. bassiana*), YYC-091 (*M. robertsii*) and ILT-01 (*I. cateniannulata*) from each genus on the study as determined by virulence assay were selected. Infection of *T. molitor* larvae was conducted as before with a constant dose of 1×10^8 conidia/g and reared in a similar condition. Larvae were sampled at 6, 12 and 24-hours post infection (hpi). Sampled larvae were fixed by glutaraldehyde 2.5% solution, dehydrated by graded ethanol series then dried using the critical-point drier with liquid carbon dioxide, sputter-coated with gold (Hayat, 1981) and then observed in a Hitachi S-3400N scanning electron microscope (Hitachi, Tokyo, Japan) at 5 kV at the college of plant protection, NWAFU, China.

Sample preparation, enzyme extraction and protein determination

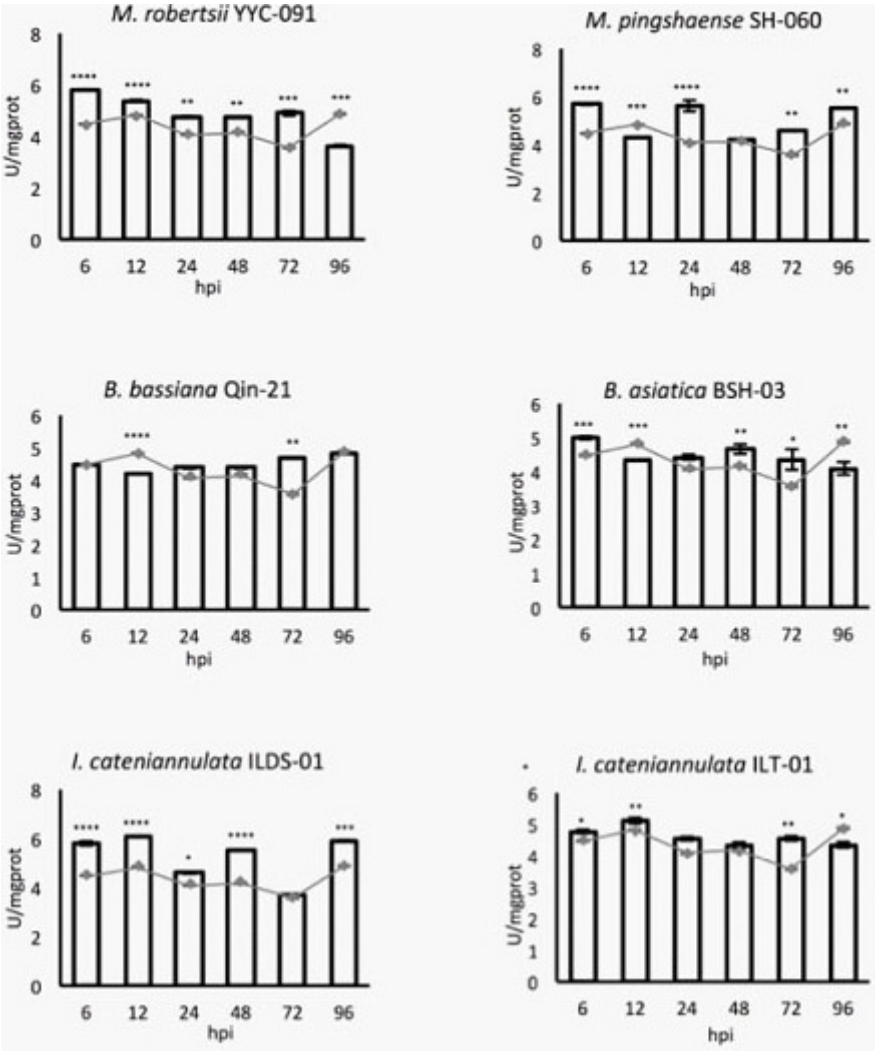

Fig. 5 : Comparative effect of different EPFs treatment over control on *T. molitor* larvae for Superoxide Dismutase (SOD) enzyme activity from 6 to 96hpi (X-axis). Enzyme activity on control group is presented by line graph and that of treatment by bar graph. Y-axis represents enzyme activity (U/mgprot). Data presents the Mean ± SEM of three replicates each with 5 larvae. Note: *, **, ***, ****, denotes significance at 5, 1, 0.1 and 0.01%, respectively (Dunnett's multiple comparisons test).

0.1M PBS solution of pH 7.4 was used in the ratio of 1:9 weight/volume for extraction which was centrifuged at 4°C for 15 minutes at 3500 rpm in an eppendorf microcentrifuge unless otherwise mentioned. The lipid layer of the supernatant fraction was removed by filtration through glass fibers at 4°C and thus collected supernatant was used as an enzyme source. The enzyme activity was tested with three independent biological replicates and each replicate consisted of 5 larvae pooled together for enzyme extraction. Crude enzymes were stored at -80 °C until further use. The protein concentration of the enzyme extract was determined through Bradford assay using Coomassie Brilliant Blue G-250 as described by Bradford (1976). Bovine Serum Albumin (BSA) was used as the reference standard. Enzyme assay kits were purchased from Jiancheng Bioengineering Institute (Nanjing, China) and the activity was estimated according to manufacturer's protocol unless otherwise mentioned.

Anti-oxidative enzymes activity assay

Catalase (CAT) activity : The CAT activity assay was estimated using the Catalase Assay Kit (A007-2) following the manufacturer's instruction. Working principle adopts the ability of CAT to decompose

Table 1 : Geographical location of the sampling sites for isolated entomopathogenic fungi used in the study.

S.No.	Name	Strain ID	Location	Coordinates
1	*Beauveria bassiana*	Qin21	China: Shaanxi: Qinling Mt, FNNR	33^0 39′ 79″ N, 107′ 47^0 14″ E
2	*Beauveria asiatica*	BSH-03	China: Sichuan: Bazhong, San Hue	31^0 70′ 05″ N, 106′ 58^0 45″ E
3	*Isaria cateniannulata*	ILT-01	China: Sichuan: Bazhong: Ling Ton	30^0 15′ 80″ N, 100′ 58^0 67″ E
4	*Isaria cateniannulata*	ILDS-01	China: Sichuan: Bazhong: Long Ding Shan	31^0 33′ 30″ N, 106′ 34^0 08″ E
5	*Metarhizium robertsii*	YYC-091	China: Sichuan: Bazhong, Yu Xi Zhen	31^0 13′ 71″ N, 106′ 60^0 13″ E
6	*Metarhizium pingshaense*	SH-060	China: Sichuan: Bazhong, San Hue	31^0 70′ 05″ N, 106′ 58^0 45″ E

FNNR = Foping National Reserve, Shaanxi, China

T. molitor larvae were infected with a constant dose of 1×10^8conidia/gm for each of six fungal strains along with control as mentioned above and samples were taken at 6, 12, 24, 48, 72 and 96 –hour post infection (hpi). Insect body of beheaded larvae were weighted and homogenized in a pre-cooled sterilized mortar and pestle. H_2O_2(hydrogen peroxide) reducing its concentration in the reaction solution corresponds to the optical density (OD) value measured at 240nm. The decrease in absorbance over a period of 1 min was recorded by Infinite 200 pro Spectrophotometer, (Tecan, Austria). One unit of CAT activity was defined as 1 µmol H_2O_2 consumed

Table 2 : Result of Probit analysis and calculated lethal dose (LD) values from dose dependent virulence assays at 18-day post infection for different isolates of EPFs against *T. molitor* larvae. The concentrations presented are conidia/g.

Fungus ID	n	df	Slope ± SE	Z	P (Z)	χ^2	P (χ^2)	LD50 (95% FL)	Lower bound	Upper bound	LC90 (95% FL)	Lower bound	Upper bound
M. pingshanse SH-060	135	2	0.834±0.065	12.755	<0.001	28.735	<0.001	2.27E+06	1.58E+06	3.19E+06	7.80E+07	4.59E+07	1.55E+08
M. robertsii YYC-091	135	2	0.541±0.067	8.077	<0.001	1.985	0.371	9.12E+04	2.76E+04	2.03E+05	2.13E+07	1.08E+07	5.56E+07
I. cateniannulata ILDS-01	135	2	0.714±0.059	12.146	<0.001	18.84	<0.001	1.24E+07	8.43E+06	1.86E+07	7.70E+08	3.69E+08	2.06E+09
I. cateniannulata ILT-01	135	2	0.747±0.063	11.953	<0.001	23.147	<0.001	2.10E+06	1.40E+06	3.04E+06	1.09E+08	6.01E+07	2.37E+08
B. asiatica BSH-03	135	2	0.376±0.052	7.269	<0.001	8.332	0.016	4.10E+07	1.99E+07	1.10E+08	1.06E+11	1.28E+10	3.90E+12
B. bassiana Qin-21	135	2	0.990±0.096	10.296	<0.001	5.514	0.063	3.45E+05	2.21E+05	4.96E+05	6.79E+06	4.40E+06	1.21E+07

(Note: FL= fiducial limits; df = number of different concentrations used for regression minus 2 (Finney, 1971); Z is the ratio of the coefficients to the standard error of the fungal concentrations)

by per mg tissue protein per second.

Superoxide Dismutase (SOD) activity : The SOD activity was estimated using the T-SOD Assay Kit (A001-2) following the manufacturer's instruction which is based on the principle that the reaction of xanthine and xanthine oxidase produces superoxide anion and oxidizes hydroxylamine to form nitrite. With the chromogenic agent, nitrite presents purple but the presence of SOD reduces the contents of superoxide anion thus decreasing the nitrite production. One SOD activity unit is the corresponding quantity of SOD required for 1 mg tissue proteins in 1 ml of reaction mixture SOD inhibition rates to 50% as monitored at 550nm.

Peroxidase (POD) activity : The POD activity was estimated using the Peroxidase Assay Kit (A084-1) following the manufacturer's instruction. The principle of measurement is based on the catalytic activity of peroxidase on the reaction of hydrogen peroxide showing the corresponding change in absorbance at 420 nm. 1 U POD activity means 1 µg substrate catalyzed by POD from 1 mg tissue protein per minute at 37°C.

Detoxification enzymes activity assay

Glutathione S-Transferase (GST) activity : GST Assay Kit (A004) was used following the manufacturer's protocol for the GST activity estimation in the sample. Assay principle is based on the ability of GST to catalyze reduced glutathione (GSH) combined with 1-chloro-2,4-dinitrobenzene (CDNB) in a certain reaction period. GSH concentration before and after is measured to reflect the linearly correlated GST activity. 1 unit of GST activity corresponds to the decrease in GSH concentration by 1µmol/L catalyzed by GST from 1 mg tissue protein per min at 37 °C. Larger the reduction in GSH concentration higher the GST activity.

Carboxylesterase(CarE) activity: *T. molitor* larvae were sampled and processed as above was homogenized with special extraction liquid in the ratio of 1:9 weight/volume provided with the Carboxylesterase Assay Kit (50T) in pre-cooled sterilized mortar and pestle, which was then centrifuged at 4°C for 30 minutes at 12000 g in an Eppendorf microcentrifuge. The supernatant thus collected was used as an enzyme source. CarE activity was measured using the same kit following the manufacturer's instruction. Where 1U enzyme activity corresponds to 1 absorption value catalyzed by 1g tissue protein per minute.

Alkaline Phosphatase (AKP) activity : AKP Assay Kit (A059-1) was used to determine AKP activity based on the principle that alkaline phosphatase can decompose disodium phenyl phosphate to free phenol and phosphoric

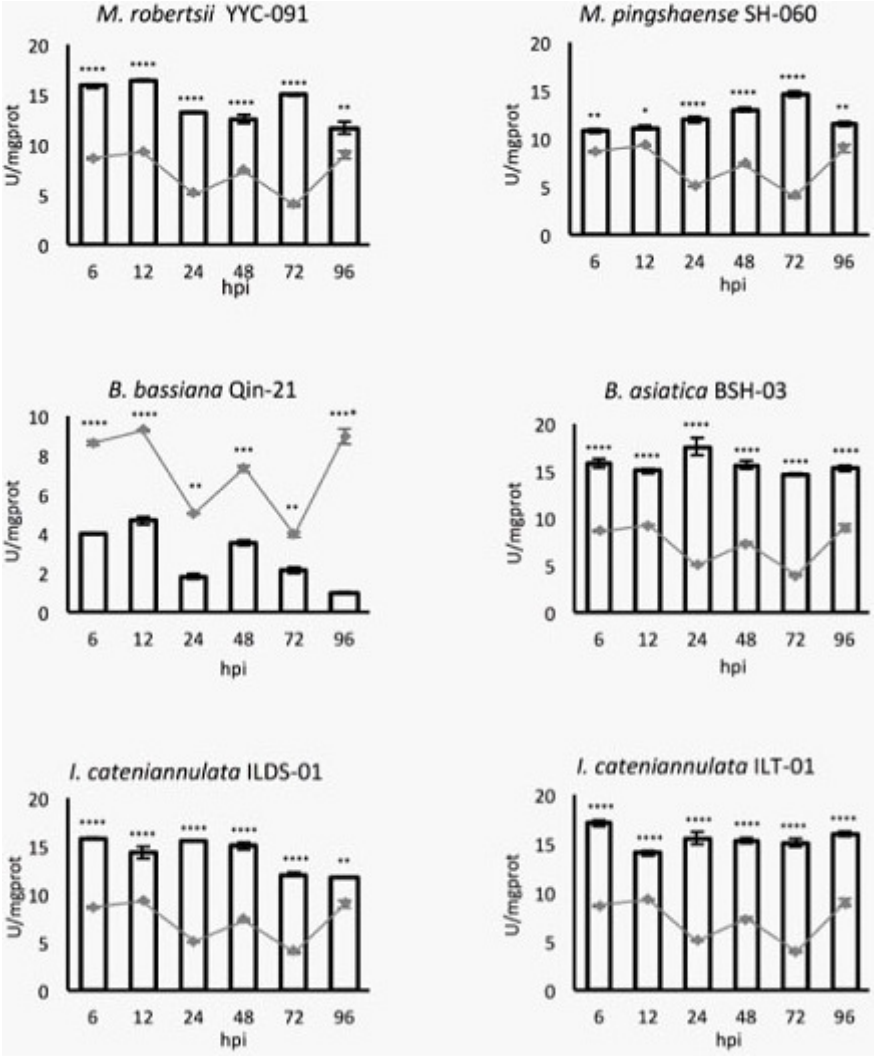

Fig. 6 : Comparative effect of different EPFs treatment over control on *T. molitor* larvae for Peroxidase (POD) enzyme activity from 6 to 96hpi (X-axis). Enzyme activity on control group is presented by line graph and that of treatment by bar graph. Y-axis represents enzyme activity (U/mgprot). Data presents the Mean ± SEM of three replicates each with 5 larvae. Note: *, **, ***, ****, denotes significance at 5, 1, 0.1 and 0.01%, respectively (Dunnett's multiple comparisons test).

acid. In alkaline solution, phenol reacts with 4-amino pyrazoline, oxidized by potassium ferricyanide to produce red quinone derivate and the degree of red measured by spectrophotometer can determine enzyme activity. 1U enzyme activity unit means 1mg phenol produced by 1g tissue per 15min.

All the enzyme activities are expressed in Enzyme Unit/gram protein (U/gprot). Experimental data for all the enzymatic activity were analyzed using one-way ANOVA and significance among the treatments were compared at $P<0.05$ using Dunnett's multiple comparisons tests using GraphPad Prism Ver. 6.0 for Windows (GraphPad Software, La Jolla California, USA).

RESULTS AND DISCUSSION

Virulence assay

EPFs have a broad host range but their virulence can differ within and between species/genus. In this context, screening test based on the laboratory assay to identify a potential candidate across available EPFs isolates is a plausible starting point before planning for the field implementation. In our study, all the six fungal isolates were found pathogenic to *T. molitor* larvae and the larval mortality rate was found positively correlated with the conidial concentration (Table 1). Different isolates showed a varying level of virulence where YYC-091 and Qin-21 were among the most virulent with LD_{50} of 9.12×10^4 and 3.4×10^5 and LD_{90} of 2.13×10^7 and 6.79×10^6 respectively. BSH-03 showed the least virulence among all the tested strains with LD_{50} of 4.1×10^7 and LD_{90} of 1.06×10^{11} and among two isolates from *Isaria:* ILT-01 showed higher virulence (Table 2).

Different isolates illustrated a different level of Mean Survival Time (MST) in different concentration of infection based on the daily mortality of larvae. MST ranges from 3.44 ± 0.04 to 11.67 ± 0.45 days for the highest concentration of inoculation (2×10^8 conidia/g) where Qin-21 could kill the larva faster than the other isolates; ILT-01 has the MST of 4.33 ± 0.07 days with no significant difference between these two strains according to Tukey's grouping. For other consecutive doses of inoculation YYC-091 showed least MST among others with 7.58 ± 0.33, 10.56 ± 0.40 and 12.53 ± 0.42 days in 2×10^7, 2×10^6 and 2×10^5 conidia/g respectively. Whereas BSH-03 showed the least virulence with highest MST of 11.67 ± 0.45, 15.22 ± 0.37 days for 2×10^8 and 2×10^7 conidia/g inoculation and ILDS-01 showed highest MST of 16.18 ± 0.30 and 16.91 ± 0.22 days for later consecutive doses of inoculation (Table 3). Superiority in virulence of isolates from *Beauveria* and *Metarhizium* over *Isaria* as found in our experiment is in accordance with (Antonio Flores, 2012; Flores-Villegas

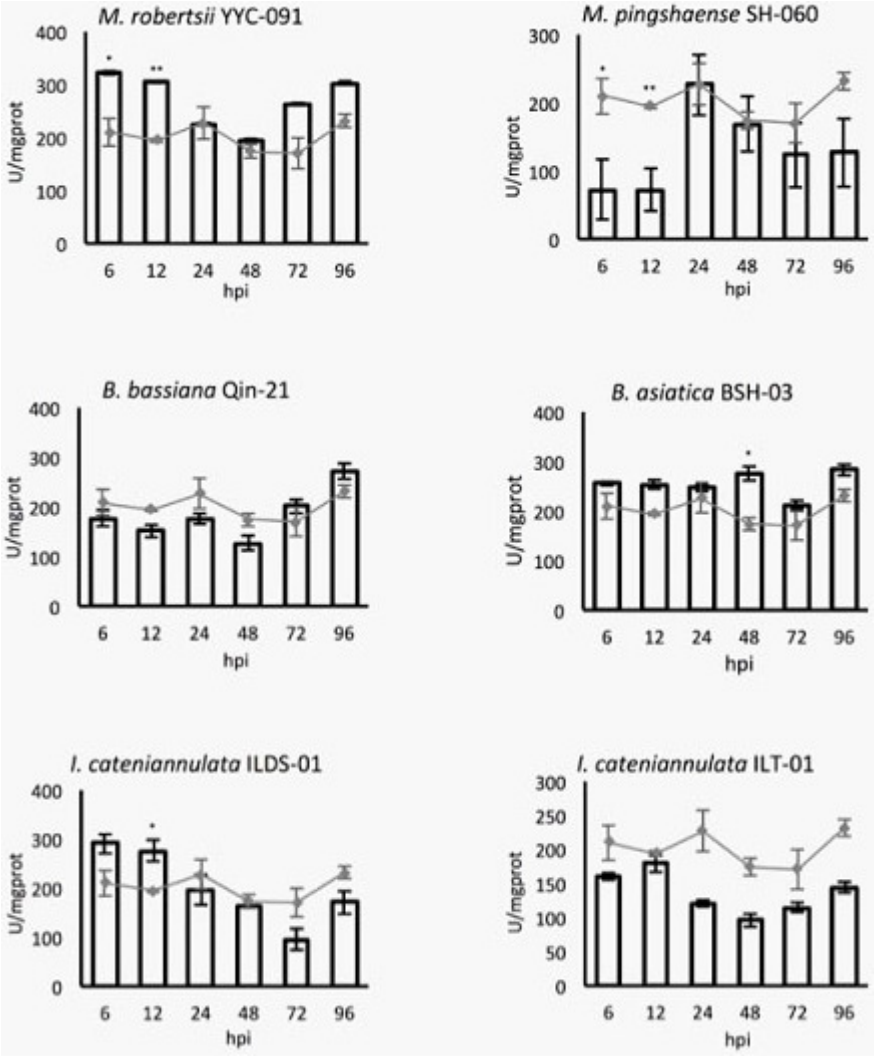

Fig. 7 : Comparative effect of different EPFs treatment over control on *T. molitor* larvae for Glutathione S-Transferase (GST) enzyme activity from 6 to 96hpi (X-axis). Enzyme activity on control group is presented by line graph and that of treatment by bar graph. Y-axis represents enzyme activity (U/mgprot). Data presents the Mean ± SEM of three replicates each with 5 larvae. Note: *, **, ***, ****, denotes significance at 5, 1, 0.1 and 0.01%, respectively (Dunnett's multiple comparisons test).

et al, 2016) tested against other insect systems.

Morphology and pathogenicity of isolates

Each isolate showed their characteristic colony color, where isolates within *Beauveria* and *Isaria* were white in color. Fungal colony on SDAY medium for *Beauveria* spp. were more dense and cottony than *Isaria* spp. For both of them, conidia were white colored in mass, round, globose shape with occasional hilum as was described by Rehner *et al* (2011). In contrast, *Metarhizium fungal* colonies were green in color, the YYC-091 isolate elucidated lighter green and the SH-060 isolate, darker green, both of them had a dense cushiony appearance. Conidia were green and round to cylindrical shaped. Their morphological characteristics were in accordance to those described by Zimmermann (2008), Fernandes *et al* (2010) and Imoulan *et al* (2017). All the isolates were pathogenic to *T. molitor* larvae (Fig. 1). Early germination was examined through SEM of the more virulent isolates from representative genera. Conidial budding started as early as 12 hpi for Qin-21 and YYC-091 whereas ILT-01 showed no budding till 24 hpi; YYC-091 showed extensive growth of germ tube on the surface of the larvae unlike other two isolates (Fig. 2). This result is on par with our earlier facts that because of early germination and better growth rates, YYC-091 and Qin-21 isolates outcompeted the ILT-01 in virulence test. Similar results have been obtained in other studies in which germination and growth rates of EPFs are considered to be substantial in the determination of virulence (Varela and Morales, 1996; Safavi *et al*, 2007).

Effect of different fungal isolates on anti-oxidative enzyme activities during infection

Anti-oxidative enzymes are an integral part of insect immune system, they play an important role in the elimination of reactive oxygen species (ROS) (Lijun *et al*, 2005). Small quantities of ROS are formed as a byproduct of aerobic respiration under normal condition (Pietta, 2000; Simmonds, 2003). But when the organism is exposed to the external interference like irradiation, infection chemicals, toxins; the production of ROS increases and can cause damage to the cell membrane, nucleotides and enzymes (Finkel and Holbrook, 2000; Büyükgüzel and Kalender, 2009). To counteract the increasing level of ROS, insects produce anti-oxidative enzymes which can detoxify the ROS (Kamala Jayanthi *et al*, 2015). In our study, total protein content in crude enzyme extract ranged from 8.22 to 11.90 mg/ml (Fig.3). CAT enzyme activity for control treatment ranged from 0.02 ± 0.002 to 0.039 ± 0.01 U/mgprot. Enzyme activity was elevated following infection for all the treatments compared to that of control, signifying the increased oxidative stress, though the change pattern over time was different between treatments. All the treatments except Qin-21 and BSH-03 illustrated dwindling pattern

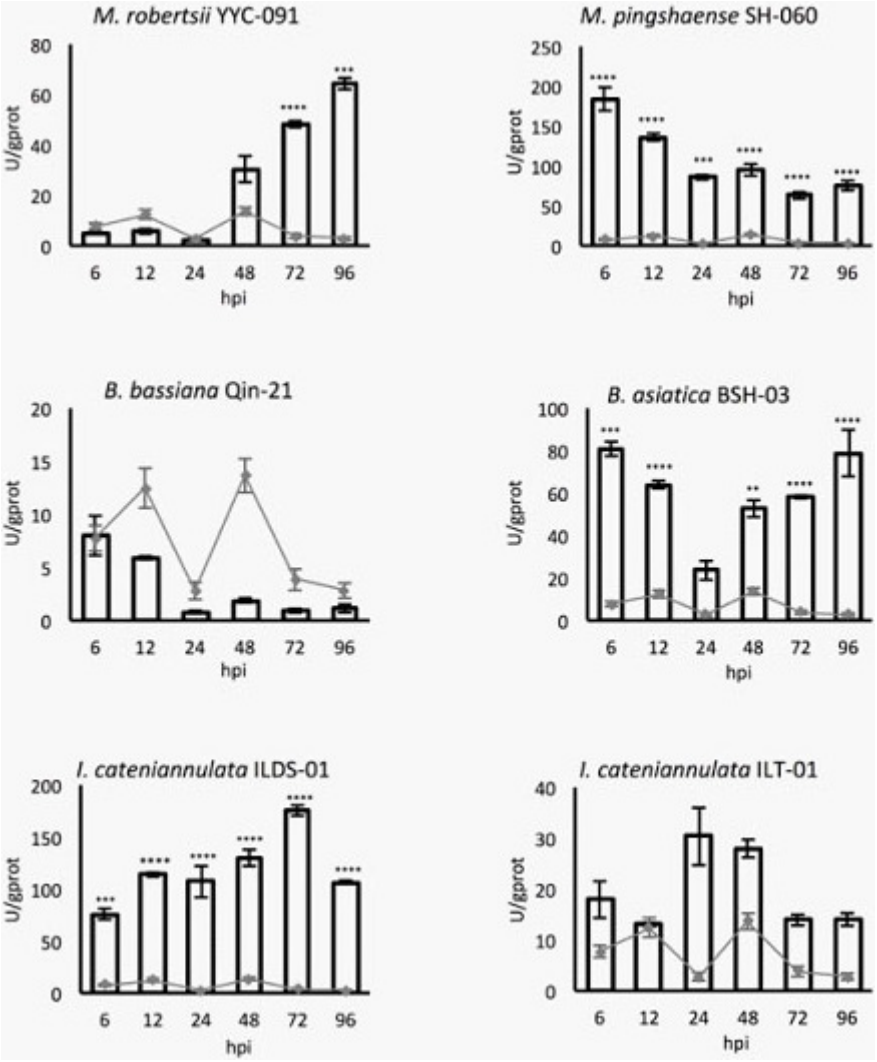

Fig. 8 : Comparative effect of different EPFs treatment over control on *T. molitor* larvae for Carboxylesterase (CarE) enzyme activity from 6 to 96hpi (X-axis). Enzyme activity on control group is presented by line graph and that of treatment by bar graph. Y-axis represents enzyme activity (U/mgprot). Data presents the Mean±SEM of three replicates each with 5 larvae. Note: *, **, ***, ****, denotes significance at 5, 1, 0.1 and 0.01%, respectively (Dunnett's multiple comparisons test).

at 96 hpi compared to earlier. CAT activity peaked significantly at 12 hpi (P=0.0292) for ILDS-01 treatment and then decreased at later time points. Significantly, upregulated activity at 48 hpi for ILT-01 and SH-060 (P=0.0027 and P=0.042, respectively) was observed which then decreased on later time points (Fig. 4).

Control treatment showed stable SOD activity with the range of 3.57 ± 0.05 to 4.87 ± 0.04 U/mgprot but different treatments showed mixed regulation within and among treatments. YYC-091 showed significant upregulation of SOD from 6 to 72 hpi, which were highly significant at 6 and 12 hpi (P<0.0001), Similarly, ILDS-01 also showed highly significant upregulation at early time points (6 and 12 hpi; P<0.0001). Qin-21 and SH-060 showed significant downregulation only at 12 hpi (P<0.0001, P=0.0002 respectively) but were highly significantly upregulated at 6 and 24 hpi (P<0.0001) in case of SH-060 (Fig. 5). Both the CAT and SOD enzyme activity did not show comparable patterns or difference in virulence of different isolates, where, CAT activity was elevated for all the treatments implying the response for the oxidative stress even the differences were trivial to all treatments throughout the time and SOD showed mixed responses between isolates and also between time points within isolates.

However, POD showed some interesting activities; control treatment was fairly stable for its activity (3.94 ± 0.13 to 9.25 ± 0.06 U/mgprot). Qin-21 treatment surprisingly down regulated POD activity with a highly significant difference at 6, 12 and 96 hpi (P<0.0001) and maintaining its significant down regulation at 24-72 hpi (P= 0.0058 to 0.0003) while all other fungal treatments caused its upregulated activity throughout the time. Highly significant upregulation (P<0.0001) for all tested time points in BSH-03 and ILT-01 treatment was observed. Furthermore, YYC-091 and ILDS-01 upregulated POD activity with a highly significant difference (P<0.0001) from 6-72 hpi and differences reduced slightly at 96 hpi with P =0.0024 and P=0.002 respectively. At the same time, SH-060 exhibited increasing effect with time till 72 hpi with highly significant differences from 24-72 hpi (P<0.0001) and effect reduced at 96 hpi (Fig. 6). This clearly illustrates the correlation between lowest MST and highest virulent of Qin-21 isolate with impairment of anti-oxidative enzyme (POD) in *T. molitor*. This finding is in accordance with the findings from Kamala Jayanthi *et al* (2015) where suppression of anti-oxidative enzymes in *Sternochetus mangiferae* by *Aspergillus flavus* was reported.

Effect of different fungal isolates on detoxification enzyme activities during infection

Detoxifying enzymes like GST, CarE and AKP play a pivotal role in insect resistance (Wang *et al*, 2016). They are regarded important in neutralizing exogenous

Table 3 : Survival analysis results of *T. molitor* larvae from time-mortality response at various concentration of infection for different isolates of EPFs.

Infection dose	Fungus ID	MST (±SE) *	95% Confidence Interval
2.00E+08	*B. bassiana* Qin-21 210E8	3.44 (0.04) ^{A}	3.36 ~ 3.53
	B. asiatica BSH-03 210E8	11.67 (0.45) ^{E}	10.79 ~ 12.54
	I. cateniannulata ILT-01 210E8	4.33 (0.07) $^{A, B}$	4.20 ~ 4.47
	I. cateniannulata ILDS-01 210E8	8.18 (0.33) ^{D}	7.54 ~ 8.82
	M. robertsii YYC-091 210E8	5.49 (0.21) $^{B, C}$	5.08 ~ 5.90
	M. pingshanse SH-060 210E8	6.76 (0.12) ^{C}	6.52 ~ 6.99
	Control	17.88 (0.08) ^{F}	17.72 ~ 18.04
2.00E+07	*B. bassiana* Qin-21 210E7	9 (0.25) ^{C}	8.51 ~ 9.49
	B. asiatica BSH-03 210E7	15.22 (0.37) ^{E}	14.50 ~ 15.94
	I. cateniannulata ILT-01 210E7	12 (0.42) ^{D}	11.18 ~ 12.82
	I. cateniannulata ILDS-01 210E7	13.8 (0.44) $^{E, D}$	12.95 ~ 14.65
	M. robertsii YYC-091 210E7	7.58 (0.33) ^{C}	6.94 ~ 8.22
	M. pingshanse SH-060 210E7	12.22 (0.39) ^{D}	11.45 ~ 13.00
	Control	17.88 (0.08) ^{F}	17.72 ~ 18.04
2.00E+06	*B. bassiana* Qin-21 210E6	11.16 (0.40) ^{C}	10.38 ~ 11.93
	B. asiatica BSH-03 210E6	15.42 (0.35) $^{D, E}$	14.74 ~ 16.11
	I. cateniannulata ILT-01 210E6	13.87 (0.38) ^{D}	13.12 ~ 14.61
	I. cateniannulata ILDS-01 210E6	16.18 (0.30) $^{E, F}$	15.58 ~ 16.77
	M. robertsii YYC-091 210E6	10.56 (0.40) ^{C}	9.77 ~ 11.34
	M. pingshanse SH-060 210E6	15 (0.39) $^{B, D}$	14.23 ~ 15.77
	Control	17.88 (0.08) ^{F}	17.72 ~ 18.04
2.00E+05	*B. bassiana* Qin-21 210E5	14.02 (0.40) $^{C, D}$	13.24 ~ 14.80
	B. asiatica BSH-03 210E5	16.33 (0.28) $^{E, F}$	15.79 ~ 16.88
	I. cateniannulata ILT-01 210E5	15.62 (0.35) $^{D, E}$	14.94 ~ 16.30
	I. cateniannulata ILDS-01 210E5	16.91 (0.22) $^{E, F}$	16.47 ~ 17.35
	M. robertsii YYC-091 210E5	12.53 (0.42) ^{C}	11.72 ~ 13.35
	M. pingshanse SH-060 210E5	15.56 (0.36) $^{D, E}$	14.84 ~ 16.27
	Control	17.88 (0.08) ^{F}	17.72 ~ 18.04

Note: MST = mean survival time (± standard error) given in days.
Kaplan-Meier survival analysis was used to estimate MST for each isolate at different concentration of infection based on daily mortality for up to 18 days. Different letters after MST indicate the groups of significances (P<0.05, Tukey-Kramer HSD test).

and endogenous toxic substances, hormone synthesis and protection from oxidative stress (Wu and Liu, 2012; Li *et al*, 2016). In our experiment, control treatment has the enzyme activity range of 170.4 ± 28.99 to 231.6 ± 12.56 U/mgprot. YYC-091 isolate caused significant early upregulation of GST activity (P=0.0335 at 6 hpi; P=0.0083 at 12hpi), which is opposite in the case of SH-060 isolate causing early significant downregulation at 6 (P=0.0125) and 12 hpi (P=0.0048). BSH-03 isolate caused continuous elevated activity throughout the timeframe but significant difference was observed only at 48hpi (P=0.0231). The opposite was the case for ILT-01 isolate treatment where activity was lowered without significant difference throughout the time (Fig. 7).

CarE showed the range of 2.8 ± 0.82 to 13.64 ± 1.59 U/gprot for control treatments. YYC-091 isolate infection increasingly upregulated enzyme activity from 48 to 96 hpi with a significant difference at 72hpi (P<0.0001) and at 96hpi (P=0.0002). All other treatments caused elevated CarE activity throughout the infection period except Qin-21 isolate which suppressed the enzyme activity from 12 to 96 hpi, but no such statistically significant difference was observed compared to control treatment (Fig. 8).

AKP activity ranged from 3.7 0.21 to 10.66 0.31 U/gprot for the control treatment. Effect of fungal infection illustrated different trends between treatments. Only Qin-21 isolate showed continuous upregulation of the AKP activity throughout the timeframe with highly significant

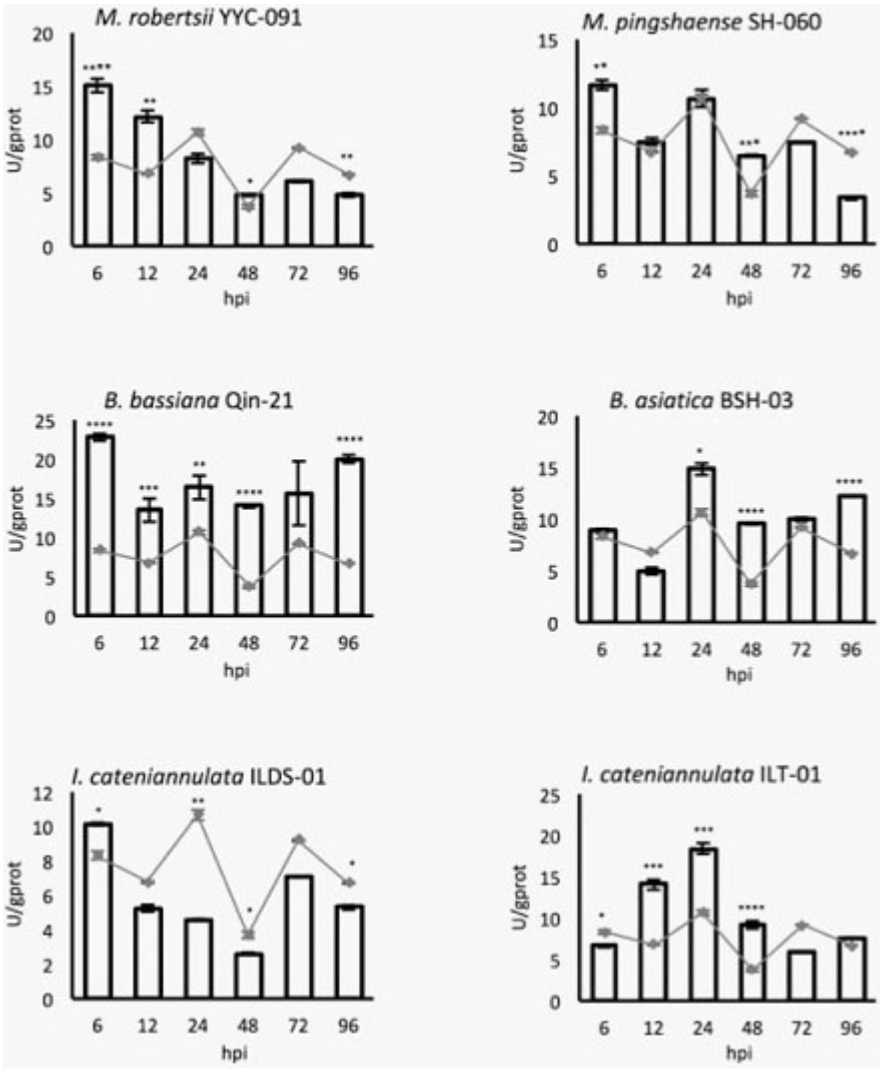

Fig. 9 : Comparative effect of different EPFs treatment over control on *T. molitor* larvae for Alkaline Phosphatase (AKP) enzyme activity from 6 to 96hpi (X-axis). Enzyme activity on control group is presented by line graph and that of treatment by bar graph. Y-axis represents enzyme activity (U/mgprot). Data presents the Mean ± SEM of three replicates each with 5 larvae. Note: *, **, ***, ****, denotes significance at 5, 1, 0.1 and 0.01%, respectively (Dunnett's multiple comparisons test).

different at 6, 48 and 96 hpi (P<0.0001). YYC-091 isolate demonstrated early significant upregulation at 6 and 12 hpi (P<0.0001 and P=0.0031 resp.) and continuous suppression during later time points leading to significant down regulation at 96 hpi (P=0.0019). ILDS-01 isolate treatment caused early up-regulation at 6 hpi (P=0.0314) and suppressed the activity in the following time points with a significant difference at 24 (P=0.0027), 48 (P=0.0334) and 96 hpi (P=0.0108) (Fig. 9).

Overall, GST did not exhibit any virulence dependent activity differences whereas CarE, a crucial detoxification enzyme in insect immunity system (Zhang *et al*, 2011a) showed up-regulation with varying differences between time points within and between isolates for the least virulent strains like SH-060, BSH-03, ILDS-01 and ILT-01. But was found suppressed without a statistical significant difference for Qin-21 treatment. YYC-091 isolate also showed dominance during the early phase (6 to 24 hpi) but reversed during later phase. These reduced activities must have contributed for their increased virulence among others.

CONCLUSION

Beauveria, *Metarhizium* and *Isaria* spp. are some of the well studied EPFs and possess a significant importance in the biological control of insect pests. Although, they vary in their pathogenicity and virulence within and between genera and their dependency lies on physiological to enzymatic ability for attachment, germination, penetration to the invasion of host immune system. Here, we assayed the virulence of 6 different phylogenetically close to distant isolates of EPFs and found that two isolates Qin-21 and YYC-091 as the most virulent among all

tested. The physiological observation and enzymatic analysis complemented the bioassay results and attributed the high virulence of YYC-091 isolate to its faster germination and extension of germ tube to penetrate the insect integument and that of Qin-21 isolate to its germination efficiency and ability to suppress POD and CarE enzyme activity thereby impairing anti-oxidative and detoxification process. This finding provides an useful insight about the underlying mechanism on mode of action and chemical mechanism during fungus-insect interaction and can be used further to improve the virulence of entomopathogenic fungi by exploiting fungal genetics knowledge, chemistry and physiology.

ACKNOWLEDGMENTS

This study was supported by NSFC grant (31670659), Special Fund for Forest Scientific Research in the Public Welfare (201404403-09), Yangling Key project of collaborative innovation (2017CXY-12)and Shaanxi Provincial Science and Technology Innovation Project (2014KTCL02-14). The authors declare no competing financial interests.

REFERENCES

Antonio Flores M. MPD, Silvia Rodriguez N, Mayra DeLa Torre M and Miguel A Ramos L (2012) Mycoinsecticide effects of *Beauveria bassiana, Metarhizium anisopliae* and *Isaria fumosorosea* on the whitefly *Bemisia tabaci* (Homoptera: Aleyrodidae) in different strata of bean. *Afr. J. Microbiol. Res.* **6**, 7246-7252.

Behie S, Zelisko P and Bidochka M (2012) Endophytic insect-parasitic fungi translocate nitrogen directly from insects to plants. *Science* **336**, 1576-1577.

Bidochka M J, Kasperski J E and Wild G A (1998) Occurrence of the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* in soils from temperate and near-northern habitats. *Canadian J. Botany* **76**, 1198-1204.

Borisade O A and Magan N (2014) Growth and sporulation of entomopathogenic *Beauveria bassiana*, *Metarhizium anisopliae*, *Isaria farinosa* and *Isaria fumosorosea* strains in relation to water activity and temperature interactions. *Biocontrol Science and Technology* **24**, 999-1011.

Bradford M M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Büyükgüzel E and Kalender Y (2009) Exposure to streptomycin alters oxidative and antioxidative response in larval midgut tissues of *Galleria mellonella*. *Pestic. Biochem. Physiol.* **94**, 112-118.

Carruthers R I, Sawyer A J and Hural K (1991) Use of fungal pathogens for biological control of insect pests. *Research and Education in the field.*

Charnley A and Collins S (2007) 10 Entomopathogenic Fungi and Their Role in Pest Control. *Environmental and Microbial Relationships* **4**, 159.

Chen Z-H, Xu L, Yang F-l, Ji G-H, Yang J and Wang J-Y (2014) Efficacy of *Metarhizium anisopliae* isolate MAX-2 from Shangri-la, China under desiccation stress. *BMC Microbiol.* **14**, 4.

Copping L G (2004) *The manual of biocontrol agents*: British Crop Protection Council, Alton.

do Nascimento Silva J, Mascarin G M, dos Santos Gomes I C, Tinôco RS, Quintela E D, dos Reis Castilho L and Freire D M G (2018) New cost-effective bioconversion process of palm kernel cake into bioinsecticides based on *Beauveria bassiana* and *Isaria javanica. Appl. Microbiol. Biotechnol.* **102**, 2595-2606.

Dubovskiy I M, Whitten M M A, Yaroslavtseva O N, Greig C, Kryukov V Y, Grizanova E V, Mukherjee K, Vilcinskas A, Glupov V V and Butt T M (2014) Can insects develop resistance to insect pathogenic fungi? *PLoS One* **9**, e60248.

Fernandes É K K, Keyser C A, Chong J P, Rangel D E N, Miller M P and Roberts D W (2010) Characterization of *Metarhizium* species and varieties based on molecular analysis, heat tolerance and cold activity. *J. Appl. Microbiol.* **108**, 115-128.

Finkel T and Holbrook NJ (2000) Oxidants, oxidative stress and the biology of ageing. *Nature* **408**, 239-247.

Finney D J (1971) *Probit Analysis :* 3rd ed: Cambridge University Press.

Flores-Villegas AL, Cabrera-Bravo M, Toriello C, Bucio-Torres MI, Salazar-Schettino PM and Córdoba-Aguilar A (2016) Survival and immune response of the Chagas vector *Meccus pallidipennis* (Hemiptera: Reduviidae) against two entomopathogenic fungi, *Metarhizium anisopliae* and *Isaria fumosorosea*. *Parasites & Vectors* **9**, 176.

Hajek A and St. Leger R (1994) Interactions between fungal pathogens and insect hosts. *Annu. Rev. Entomol.* **39**, 293-322.

Han SK, Lee D, Lee H, Kim D, Son HG, Yang J-S, Lee S-JV and Kim S (2016) OASIS 2 : online application for survival analysis 2 with features for the analysis of maximal lifespan and healthspan in aging research. *Oncotarget* **7**, 56147-56152.

Hayat M A (1981) *Principles and techniques of electron microscopy. Biological applications*, New York: Edward Arnold.

Imoulan A, Hussain M, Kirk PM, El Meziane A and Yao Y-J (2017) Entomopathogenic fungus *Beauveria* : Host specificity, ecology and significance of morpho-molecular characterization in accurate taxonomic classification. *J. Asia-Pacific Entomol.* **20**, 1204-1212.

Inglis GD, Johnson DL and Goettel MS (1997) Field and laboratory evaluation of two conidial batches of *Beauveria bassiana* (Balsamo) Vuillemin against grasshoppers. *The Canadian Entomologist* **129**, 171-186.

Kamala Jayanthi PD, Arthikirubha A, Vivek K, Ravindra MA, Selvakumar G and Abraham V (2015) *Aspergillus flavus* impairs antioxidative enzymes of *Sternochetus mangiferae* during mycosis. *J. Invertebr. Pathol.* **124**, 73-77.

Kepler RM, Humber RA, Bischoff JF and Rehner S A (2014) Clarification of generic and species boundaries for *Metarhizium* and related fungi through multigene phylogenetics. *Mycologia* **106**, 811-829.

Khan S, Guo L, Maimaiti Y, Mijit M and Qiu D (2012) Entomopathogenic fungi as microbial biocontrol agent. *Molecular Plant Breeding* **3**, 63-79.

Kim JC, Lee MR, Kim S, Lee SJ, Park SE, Nai Y-S, Lee GS, Shin TY and Kim JS (2018) *Tenebrio molitor* mediated entomopathogenic fungal library construction for pest

management. *J. Asia-Pacific Entomol.* **21**, 196-204.

Klingen I and Haukeland S (2006) The soil as a reservoir for natural enemies of pest insects and mites with emphasis on fungi and nematodes. *An ecological and Societal approach to biological control.* Springer, 145-211.

Li X, Liu Q, Lewis EE and Tarasco E (2016) Activity changes of antioxidant and detoxifying enzymes in *Tenebrio molitor* (Coleoptera: Tenebrionidae) larvae infected by the entomopathogenic nematode *Heterorhabditis beicherriana* (Rhabditida: Heterorhabditidae). *Parasitol. Res.* **115**, 4485-4494.

Lijun L, Xuemei L, Yaping G and Enbo M (2005) Activity of the enzymes of the antioxidative system in cadmium-treated *Oxya chinensis* (Orthoptera Acridoidae). *Environ. Toxicol. Pharmacol.* **20**, 412-416.

Masoudi A, Koprowski J l, Bhattarai U R and Wang D (2017) Elevational distribution and morphological attributes of the entomopathogenic fungi from forests of the Qinling Mountains in China. *Appl. Microbiol. Biotechnol.* **102**, 1483-1499.

Moura Mascarin G, Biaggioni Lopes R, Delalibera Í, Kort Kamp Fernandes É, Luz C and Faria M (2018) Current status and perspectives of fungal entomopathogens used for microbial control of arthropod pests in Brazil. *J. Invertebr. Pathol.*(in press).

Negrete González D, Ávalos Chávez MA, Lezama Gutiérrez R, Chan Cupul W, Molina Ochoa J and Galindo Velasco E (2018) Suitability of *Cordyceps bassiana* and *Metarhizium anisopliae* for biological control of *Cosmopolites sordidus* (Germar) (Coleoptera: Curculionidae) in an organic Mexican banana plantation: laboratory and field trials. *J. Plant Dis. Prot.* **125**, 73-81.

Nie NH, Bent DH and Hull CH (1970) *SPSS: Statistical package for the social sciences*. McGraw-Hill New York.

Payne C (1988) Pathogens for the control of insects: where next? *Phil. Trans. R. Soc. Lond. B* **318**, 225-248.

Pedrini N (2017) Molecular interactions between entomopathogenic fungi (Hypocreales) and their insect host: Perspectives from stressful cuticle and hemolymph battlefields and the potential of dual RNA sequencing for future studies. *Fungal Biol.*

Pietta P-G (2000) Flavonoids as antioxidants. *J. Nat. Prod.* **63**, 1035-1042.

Rehner SA, Minnis AM, Sung G-H, Luangsa-ard JJ, Devotto L and Humber RA (2011) Phylogeny and systematics of the anamorphic, entomopathogenic genus *Beauveria*. *Mycologia* **103**, 1055-1073.

Robledo-Monterrubio M, Alatorre-Rosas R, Viniegra-González G and Loera O (2009) Selection of improved *Beauveria bassiana* (Bals.) Vuill. strains based on 2-deoxy-D-glucose resistance and physiological analysis. *J. Invertebr. Pathol.* **101**, 222-227.

Safavi S A, Shah F A, Pakdel A K, Reza Rasoulian G, Bandani A R and Butt T M (2007) Effect of nutrition on growth and virulence of the entomopathogenic fungus *Beauveria bassiana*. *FEMS Microbiol. Lett.* **270**, 116-123.

Sasan R K and Bidochka M J (2012) The insect pathogenic fungus *Metarhizium robertsii* (Clavicipitaceae) is also an endophyte that stimulates plant root development. *Am. J. Bot.* **99**, 101-107.

Serebrov V V, Gerber O N, Malyarchuk A A, Martemyanov V V, Alekseev A A and Glupov V V (2006) Effect of entomopathogenic fungi on detoxification enzyme activity in greater wax moth *Galleria mellonella* L. (Lepidoptera, Pyralidae) and role of detoxification enzymes in development of insect resistance to entomopathogenic fungi. *Biology Bulletin* **33**, 581.

Simmonds M S (2003) Flavonoid–insect interactions: recent advances in our knowledge. *Phytochemistry* **64**, 21-30.

Sun B-D and Liu X-Z (2008) Occurrence and diversity of insect-associated fungi in natural soils in China. *Applied Soil Ecology* **39**, 100-108.

Varela A and Morales E (1996) Characterization of Some *Beauveria bassiana* Isolates and Their Virulence toward the Coffee Berry Borer *Hypothenemus hampei*. *J. Invertebr. Pathol.* **67**, 147-152.

Vilcinskas A and Götz P (1999) Parasitic Fungi and their Interactions with the Insect Immune System. In: Baker JR, Muller R and Rollinson D (eds) *Adv. Parasitol.*: Academic Press, 267-313.

Wang Z, Zhao Z, Cheng X, Liu S, Wei Q and Scott I M (2016) Conifer flavonoid compounds inhibit detoxification enzymes and synergize insecticides. *Pestic. Biochem. Physiol.* **127**, 1-7.

Wu H-D and Liu Q-Z (2012) Antioxidative responses in *Galleria mellonella* larvae infected with the entomopathogenic nematode *Heterorhabditis* sp. *beicherriana*. *Biocontrol Science and Technology* **22**, 601-606.

Zhang B, Helen HS, Wang J-J and Huai L (2011a) Performance and enzyme activity of beet armyworm *Spodoptera exigua* (Hübner)(Lepidoptera: Noctuidae) under various nutritional conditions. *Agricultural Sciences in China* **10**, 737-746.

Zhang B, Helen HS, Wang J-j and Liu H (2011b) Performance and enzyme activity of beet Armyworm *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) under various nutritional conditions. *Agricultural Sciences in China* **10**, 737-746.

Zimmermann G (2008) The entomopathogenic fungi *Isaria farinosa* (formerly Paecilomyces farinosus) and the *Isaria fumosorosea* species complex (formerly *Paecilomyces fumosoroseus*): biology, ecology and use in biological control. *Biocontrol Science and Technology* **18**, 865-901.