Universal Journal of Plant Science 5(2): 17-23, 2017
DOI: 10.13189/ujps.2017.050201 http://www.hrpub.org

Susceptibility, Antioxidant Defense, and Growth Inhibitory Response of *Rhynchophorus ferrugineus* Olivier (Coleoptera: Curculionidae) against the Virulence of *Metarhizium anisopliae* Isolates

Abid Hussain, Muhammad Rizwan-ul-Haq, Ahmed Mohammed AlJabr*

Laboratory of Bio-Control and Molecular Biology, Department of Arid Land Agriculture,
College of Agricultural and Food Sciences, King Faisal University, Kingdom of Saudi Arabia

Abstract *Rhynchophorus ferrugineus*, commonly referred to as red palm weevil (RPW), is an invasive Indian sub-continent pest that infests date palms in the Arabian peninsula and dates-growing countries. In an effort to manage red palm weevil infestations, exotic isolates of entomopathogenic fungi, especially *Metarhizium anisopliae* were procured from different sources for possible use against red palm weevil larvae. In this study, we evaluated the virulence attributes including the viability and relative hydrophobicity of the spores of *M. anisopliae* isolates 8453, 7234 and 406; and their impact on the host susceptibility, growth and antioxidant defense. Results indicated that isolate 8453 had 20.64 % higher relative hydrophobicity compared with the least virulent isolate 406 that showed the highest LT_{50} value (10.90 days). The feeding performance experiment revealed significant differences in ECI and ECD indexes. The most virulent isolate 8453 (LT_{50} = 7.55 days) established in the current study tremendously declined ECI (26.15 %) and ECD (39.84 %) indexes compared to control treatment larvae. However, the least virulent isolate 406 (LT_{50} = 10.90 days) could only reduce 7.38 % and 12.06 % of ECI and ECD indexes, respectively. Furthermore, the virulent isolate 8453 established in the current study successful imparted 100 % larval mortality compared with other tested isolates of *M. anisopliae.* Host antioxidant defense from hemolymph, gut and fat was explored after 24 h of infection by qRT-PCR. The quantification of *catalase* and *peroxidase* genes revealed significant differences in their expressions. Overall, the least virulent isolate (406) failed to induce the expression of *catalase* and *peroxidase*. However, isolate 8453 greatly induced the expression of studied antioxidant genes. These results indicated that *M. anisopliae* isolate 8453 seems to be a promising bio-control agent against the infestations of red palm weevils.

Keywords Antioxidant Defense, Bio-control, Entomopathogenic Fungi, Hydrophobicity, Red Palm Weevil, Virulence

1. Introduction

Red Palm Weevil, *Rhynchophorus ferrugineus* Olivier (Coleoptera, Curculionidae), is a serious pest of numerous palm species in Asia, Africa, Central America, Caribbean, Oceania and Europe [1]. Red palm weevil status as a pest elevated considerably since its invasion in the Arabian Peninsula on date palms. Heavy infestations of red palm weevils are mainly responsible for the destruction of palms that worth millions of dollars annually.

Grubs of red palm weevil feeding inside the palm are the main destructive stage. They chew the soft, tender tissues of the infested palm. This cryptic mode of feeding made red palm weevil management challenging for the farmers and date palm growers. A great variety of synthetic insecticides, including organophosphates [2–6], carbamates [4,5,7], pyrethroids [4,5,8], and new chemistry insecticides [5,6,9], by various methods [3,5,8,10,11], have been tried to manage the populations of *R. ferrugineus*. Although several insecticides from these groups are found to be potent, however, applicator safety, environmental pollution and development of insecticide resistance limit their efficacy against red palm weevils [1,12]. Integration of entomopathogenic fungi in the integrated pest management program for the management of red palm weevils could minimize the reliance on environmentally harmful synthetic pesticides. In addition, entomopathogenic fungi are ready-made elements of red palm weevil IPM due to their compatibility with other biological [13–15], and chemical agents [16–19].

Entomopathogenic fungi remained the subject of many insect pest species belonging to various orders, especially Blattodea [20], Diptera [21], Coleoptera [22,23], Hemiptera [24], Homoptera [25], Hymenoptera [26], Isoptera [16,27,28], Lepidoptera [29], Orthoptera [30] and Thysanoptera [31]. Until now, over nine thousand isolates of entomopathogenic fungi belonging to different genera are available for research purpose for the scientists [32]. In the past, a number of research investigations were conducted to screen the most virulent isolates against red palm weevils [22,23,33–37]. Unfortunately, no product containing entomopathogenic fungi for red palm weevil control is registered anywhere. The reasons for the unavailability of these products are because of the limited number of studies on pathogen screening and host defense throughout the world. It was the aim of the current research to investigate the virulence of exotic isolates of *M. anisopliae* by mortality, growth indices and antioxidant mechanism of red palm weevils.

2. Materials and Methods

2.1. Insect Rearing

Adults of red palm weevil were collected from the pheromone traps set up in the date palm plantations in Al-Ahsa, Kingdom of Saudi Arabia. The trapped red palm weevils were taken to the laboratory and placed in perforated cages (57.5 × 29 × 58 cm) provided with pineapples and maintained at 16-h L:D photoperiod, 75% ± 5% relative humidity and 30 ± 1 °C. The hatched larvae were reared in a cage until molt to second instar larvae. Newly molted second instar red palm weevil larvae were maintained in perforated plastic cups on artificial diet standardized in our previous laboratory experimentation at 16-h L:D photoperiod, 75% ± 5% relative humidity and 30 ± 1 °C [22].

2.2. Entomopathogenic Fungal Isolates and Their Preparations

Isolates of entomopathogenic fungi, *Metarhizium anisopliae* sensu lato 7234 (Origin = USA) and 8453 (Origin = Turkey) tested in the current study were procured from the USDA-ARS cultures collection service designated as "Agricultural Research Service collection of Entomopathogenic Fungal Cultures" (ARSEF). However, isolate 406 originally isolated from soil in China was procured from the cultures collection center, South China Agricultural University, Guangzhou, China. All the isolates were cultured in complete darkness on potato dextrose agar (Oxoid, Hampshire, UK) and maintained at 25 ± 0.5 °C, and 70% ± 5% RH. After 24 days of incubation, conidia were dislodged from the petri plates using 0.05 % Tween 80 (Sigma Aldrich, UK). Neubauer hemocytometer (Wertheim, Germany) under the microscope (×400) was used to make the required concentration (1×10^7 spores/ml) of each isolate separately.

2.3. Virulence Screening Methodologies

2.3.1. Viability of the Spores

Twenty-four-days old, the culture of each fungal isolate was used to evaluate the viability. Fifty-microliter suspension at a concentration of 1×10^7 spores/ml of each isolate was pipetted on PDA Petri plates (115 mm × 20 mm). Each experimental unit (petri plate) after ceiling with parafilm was incubated at 25 ± 0.5 °C, and 70% ± 5% RH in complete darkness. For each treatment, five petri plates representing five replicates were prepared likewise. After 16 h of incubation, the viability of the spores of each experimental unit was calculated by counting both un-germinated and germinated spores under the compound microscope (×400) from ten different fields of vision from each petri plate. The previously reported criterion that was based on the germ tube length was used for spore viability [38]. One-way ANOVA was used to analyze percent germination data of each isolate. The significant differences among all the tested isolates were compared by Fisher's LSD test [39].

2.3.2. Relative Hydrophobicity of the Spores

The similar aged (24-day-old) culture of each isolate was used to determine the relative hydrophobicity by aqueous-solvent partitioning assay [40]. In brief, spores of each isolate were suspended in 0.1 M KNO_3.at a concentration of 1×10^7 spores/ml. The optical density (OD_{total}) of each tested isolates was recorded at 660 nm using a microplate reader. A specific quantity (6 ml) of each isolate suspension was transferred to a universal bottle already having 2 ml of hexadecane. The mixture was agitated for 20 s. For aqueous phase separation, the mixture was shifted into a separation funnel. Subsequently, the optical density of the aqueous phase (OD_{aq}) at 660 nm was recorded using a microplate reader. The relative hydrophobicity of the spores of each isolate was calculated using the equation mentioned in our previous experimentation [22]. Five readings from five different preparations were recorded to get five independent replicates. The relative hydrophobicity data were analyzed by one-way ANOVA and their means were compared by Fisher's LSD test [39].

2.3.3. Laboratory Pathogenicity Bioassays

Susceptibility of the mid-aged newly molted eighth-instar *R. ferrugineus* larvae was evaluated using immersion method. In brief, 25 mid-aged newly molted red palm weevil larvae were individually dipped for 10 s in a glass beaker having 30 ml of each isolates suspension separately at a concentration of 1×10^7 spores/ml under the laminar airflow cabinet. The control treatment larvae were dipped into a 0.05 % Tween

80 solution. Infected and control treatment larvae were incubated individually into a perforated 400 ml plastic cups provided with a measured quantity of artificial diet. Each experimental unit was incubated at 16-h L:D photoperiod, 75% ± 5% relative humidity and 30 ± 1 °C. Five replicates were prepared likewise. Mortality data were recorded daily until 15-days of post-exposure. The cadavers of dead larvae were shifted under laminar airflow cabinet into a sterilized petri plate provided with wet filter paper to promote fungal outgrowths. The fungal outgrowths were observed under the compound microscope to affirm the cause of larval mortality. Time-mortality-response bioassays were repeated at different time occasions. Dead larvae in the control treatment were adjusted by commonly used Abbott's formulae [41]. The corrected angularly transformed cumulative mortality data were analyzed by repeated measure ANOVA. The significant differences among means were compared by Fisher's LSD test [42]. The time-mortality-response (LT_{50}) for each isolate was calculated with the help of POLO software by probit analysis [43].

2.4. Impact of *M. Anisopliae* Isolates on the Larval Growth of *Rhynchophorus ferrugineus*

Twenty-five newly molted eighth-instar *R. ferrugineus* larvae were dipped in the spore suspension (1×10^7 spores/ml) of each tested isolate for 10 s under the laminar airflow cabinet. Each larva from all the treatments (control and infected) was separately fed for 72 h on a measured artificial diet in perforated plastic cups (400 ml capacity). All the experimental units were incubated at 16-h L:D photoperiod, 75% ± 5% relative humidity and 30 ± 1 °C. Five replicates with each prepared on different dates were prepared. Final larval weights, frass produced during the experimentation and remaining artificial diets after 72 h were weighted to calculate efficacy of conversion of ingested food and efficacy of conversion of digested food on dry matter basis [29]. Feeding performance data were analyzed by one-way ANOVA. However, variations among their indices were compared by Fisher's LSD test [39].

2.5. Exploring the Host Antioxidant Defense against the Infection of *M. anisopliae* Isolates

The quantitative expression patterns of antioxidant genes, including *catalases* and *peroxidases* from eighth-instar red palm weevil larvae were evaluated to gain antioxidant defense insights. The newly molted eighth-instar control (Tween 80) and infected (1×10^7 spores/ml) larvae were individually fed on artificial diet for 24 h in perforated plastic cups at 16-h L:D photoperiod, 75% ± 5% relative humidity and 30 ± 1 °C. Five replicates, each with one larva was prepared separately on different dates from different generations. After 24 h, larvae were dissected in saline to separate gut, fat and hemolymph. The grinding of each sample was done in a mortar and pestle using liquid nitrogen. Total RNA from the gut, fat and hemolymph was extracted separately for each experimental unit by Qiagen RNeasy Plus Universal Mini Kit. A Clontech commercially available First strand cDNA synthesis kit was used to reverse transcribed the total RNA of each sample. The quantification of *catalase* (Forward 5'-TGGTTATGGCTCCCACACAT-3' & Reverse 5'-CCGGATCGCTTCCTGCTAAT-3') and *peroxidase* (Forward 5'-TGATGGTTGTAACCGGGAGG -3' & Reverse 5'- GCCCATATCAAGTGCATGCTT-3') was done by specific primers in CFX96 Real-Time System (Bio-Rad) using SYBR® Premix Ex Taq™ II kit from Clontech. *Beta-Actin* (Forward 5'-AAAGGTTCCGTTGCCCTGAA-3' & Reverse 5'-TGGCGTACAAGTCCTTCCTG-3') was used as a housekeeping gene to normalize the expression of target genes i.e. *catalase* and *peroxidase*. The expression values of target genes were calculated by following the Livak and Schmittgen methodology [44]. The relative fold expressions of testing genes from hemolymph, gut and fat were analyzed by two-factor CRD (Completely Randomized Design) and their variations were compared by Fisher's LSD test [39].

3. Results

3.1. Pathogen Virulence-related Characteristics of *M. anisopliae* Isolates

The virulence of the tested isolates exhibited variations resulting significant differences in relative hydrophobicity ($F = 20.8$; df = 2, 12; $p < 0.0001$) and LT_{50} values ($F = 19.3$; df = 2, 12; $p < 0.0001$). Isolate 8453 exhibited the highest relative hydrophobicity that ultimately led to the reduction in lethal time to impart 50 % mortality (LT_{50}) as shown in table 1. The least virulent isolate (406) established in the current study produced spores with low hydrophobicity that ultimately led to the highest LT_{50} values (Table 1). However, the viability of all the tested isolates remained significantly at the same level and we have observed a non-significant difference among the isolates of *M. anisopliae* ($F = 0.12$; df = 2, 12; $p = 0.8883$).

Table 1. Virulence-related characteristics of the isolates of *M. anisopliae*

Isolate	Germination (%)	Relative hydrophobicity	LT_{50} (days)
M. anisopliae 406	96.40 ± 1.21^{NS}	69.20 ± 2.20^{c}	10.90 ± 0.44^{a}
M. anisopliae 7234	96.20 ± 0.80^{NS}	80.40 ± 1.66^{b}	9.68 ± 0.32^{b}
M. anisopliae 8453	95.80 ± 0.49^{NS}	87.20 ± 2.08^{a}	7.55 ± 0.39^{c}

3.2. Larval Susceptibility of Red Palm Weevils against the Isolates of *M. anisopliae*

Larval mortality tests (Figure 1, presented as corrected cumulative percent mortality at specific time intervals) showed that all the isolates (F = 57.56; df = 2, 24; $p < 0.0001$), at all the mentioned time intervals (F = 742.16; df = 2, 24; $p < 0.0001$) exhibited significant larval mortality. In addition, we have observed significant differences in their interaction (F = 4.57; df = 2, 24; p = 0.0069). Among all the isolates, only 8453 showed 100 % mortality during the course of the whole experimentation (Figure 1). In contrary, isolate 406 like isolate 7234 failed to show 100 % mortality and remained statistically at the lowest level at all the mentioned time intervals (Figure 1).

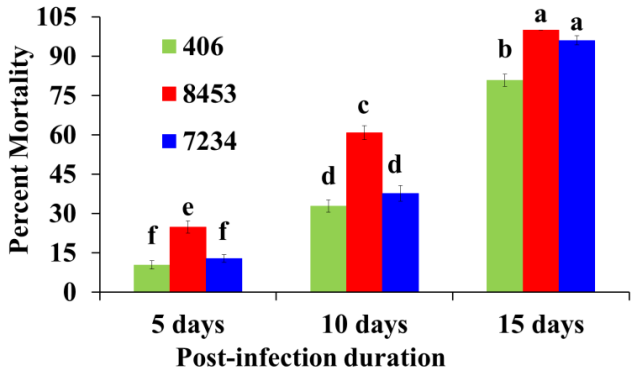

Figure 1. Corrected cumulative percent larval mortality at different time intervals (Fisher's LSD test, α = 0.05).

3.3. Larval Feeding Performance against the Infection of the Isolates of *M. anisopliae*

Infection of the isolates of *M. anisopliae* against red palm weevils by immersion greatly disturbed the larval growth (Table 2). The ECI index of the tested larvae upon infection with the isolates of *M. anisopliae* differed significantly (F = 45.40; df = 3, 16; $p < 0.0001$). The most virulent isolate imparted the highest reduction (26.15 %) in ECI compared to the control treatment (0.05 % Tween 80) larvae. On the other hand, the least virulent isolate 406 caused a 7.38 % reduction in ECI compared to control treatment larvae. However, isolate 7234 remained intermediate resulting 13.56 % reduction in ECI index compared to control treatment larvae.

Table 2. Feeding performance of *Rhynchophorus ferrugineus* larvae against different isolates of *M. anisopliae*

Isolate	ECI (%)	ECD (%)
Control (Tween 80)	18.38 ± 0.09^{a}	34.25 ± 0.17^{a}
M. anisopliae 406	17.03 ± 0.30^{b}	30.13 ± 0.87^{b}
M. anisopliae 7234	15.89 ± 0.14^{c}	26.75 ± 0.18^{c}
M. anisopliae 8453	13.58 ± 0.49^{d}	20.62 ± 0.90^{d}

ECD represents Efficacy of conversion of digested food, while ECI represents Efficacy conversion of ingested food into biomass. The numerical values (n = 25) represent the means of five independent replicates prepared at different time intervals. Nutritional indices means ± SE within each column followed by different letter(s) are statistically significant different (Fisher's LSD test, α = 0.05)

Similarly, ECD index calculated from each treatment also showed significant differences (F = 8.19; df = 3, 16; $p < 0.0001$). Among all the tested isolates, 406 remained statistically at the lowest level, resulting in the lowest reduction (12.06 %) in ECD index compared to control treatment larvae (Table 2). On the other hand, the isolate 8453 showed the least LT_{50} values (7.55 days), greatly reduced the ECD index resulting in the highest reduction (39.84 %) in ECD index compared to control larvae. Isolate 7234 imparted 21.90 % reduction in ECD index compared to the larvae immersed in 0.05 % Tween 80 solution as a control treatment (Table 2).

3.4. Antioxidant Defense of Red Palm Weevils against *M. anisopliae* Infection

Isolate 8453 established as the most virulent in the current study markedly induced the expression of *catalase* in the target larval instar after 24 h of infection. The *catalase* expression pattern among different body components (F = 798.40; df = 2, 36; $p < 0.0001$), against the infection of different isolates (F = 1069.16; df = 2, 36; $p < 0.0001$), and their interactions (F = 182.42; df = 4, 36; $p < 0.0001$), exhibited significant differences. Among all the components, gut exhibited the highest expression of *catalase*. On the other hand, the lowest expression of *catalase* was observed from the hemolymph of red palm weevil larvae (Figure 2). Overall, the isolate with the least LT_{50} (7.55 days) induced the highest expression of *catalase*, and isolate 406 negligibly induced the expression of *catalase* in the target larval instar.

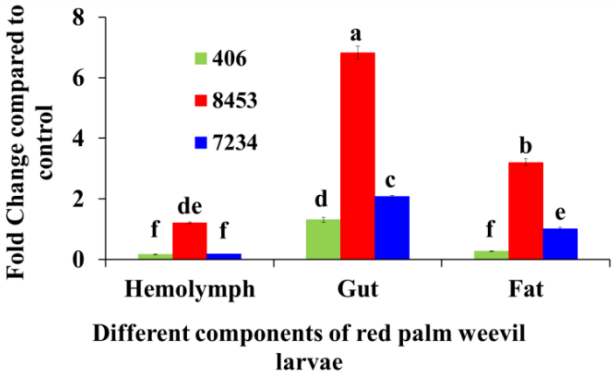

Figure 2. Relative folds expression pattern of *Rhynchophorus ferrugineus catalase* in response to the infection of the isolates of *M. anisopliae*. Means ± SE of *catalase* with different letter(s) are statistically significant different (Fisher's LSD, α = 0.05)

Expression of *peroxidase* in three different components (F = 154.79; df = 2, 36; $p < 0.0001$), upon infection with different isolates (F = 353.83; df = 2, 36; $p < 0.0001$), and their interactions (F = 34.46; df = 4, 36; $p < 0.0001$), differ significantly after 24 h post-infection among target larval instars. Overall, hemolymph exhibited the highest expression of *peroxidase* from all the experimental units (Figure 3). *Peroxidase* expression from gut remained statistically at par with fat. However, the infection of the least virulent isolate (406) could not induce the highest

expression and remained significantly at the lowest level compared to the highest virulent isolate (8453) and moderately virulent isolate (7234).

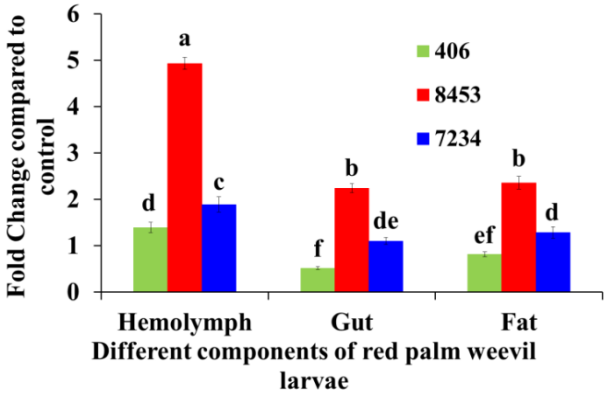

Figure 3. Relative folds expression pattern of *Rhynchophorus ferrugineus peroxidase* in response to the infection of the isolates of *M. anisopliae*. Means ± SE of *peroxidase* with different letter(s) are statistically significant different (Fisher's LSD, α = 0.05)

4. Discussion

Screening the most virulent isolate is the foundation of successful pest management strategy for the eco-friendly development of microbial pesticide for sustainable crop production. Current study disclosed that the spores with higher hydrophobicity and viability greatly disturbed the feeding performance and host antioxidant defense that ultimately lead to the 100 % larval mortality.

The Success or failure of the fungal isolate is mapped through the virulence determining attributes of the fungi and host defense mechanisms. A fruitful infection starts with the attachment of spores with the host cuticle. Previous research investigations rightly demonstrated that hydrophobicity of the spores is of prime importance for conidial adhesion that leads towards mycosis [32]. In the current study, we found variations in relative hydrophobicity of the spores of the tested isolates of *M. anisopliae*. The spores of a most virulent isolate 8453 exhibited 20.64 % higher relative hydrophobicity compared to the least virulent isolate 406 enabling us to suggest that the higher estimates of relative hydrophobicity directly related to the host pathogenicity. Our findings further strengthened from previous investigations in which virulent transgenic strain of *B. bassiana* was developed that showed higher expression of genes encoding hydrophobicity of the spores [45]. In addition, our results are in agreement with previous findings against other genera of entomopathogenic fungi, including *Beauveria bassiana* [23], and *Isaria fumosorosea* [22]. Another important virulence attribute studied in the current investigation was the germination of the spores. However, we found over 95 % viability from all the tested isolates enabling us to suggest that viability is an important virulence parameter, but high viability did not mean highly virulent isolate as depicted from the viability results of less virulent isolate 406. These results corroborate with our previous results against other isolates of entomopathogenic fungi [22,23].

Robust indices calculated in the feeding performance experiment unveiled significant differences in ECI and ECD indexes. The most virulent isolate 8453 established in the current study tremendously declined these indexes of growth. The reason for reduced indexes might because of deficiency in available food reserves that may arise due to 1) low food intake due to infection, 2) high energy utilization for host defense instead of growth to combat pathogen attack. Enhanced expression of *peroxidase* and *catalase* among red palm weevil larvae infected with 8463 depicted in the current study and low food intake in nutritional experiment provides the experimental evidence for these two conditions. These inferences enabled us to describe that the major portion of the larval food intake transformed into energy to combat the attack of invading pathogen, and less energy remained to channel larval growth. The earlier research investigations against lepidopteran pests also claimed the reductions in these indexes due to fungal infections [29,46], and botanicals [47]. In addition, isolates of *B. bassiana* also exhibited similarly disturbed growth and antioxidant defense pattern among target pest red palm weevils [23]. Similarly, reduced ECI and ECD indexes due to virulent pathogens [22,23,29,46], potent botanicals [47–49], and toxic insecticides [12], revealed that host spent most of his energy reserves to combat attacks through physiological activities. In these situations, the host became vulnerable to any attack that ultimately leads towards host mortality. This may clearly explain the difference in mortality results of red palm weevil larvae against different infections. The superiority of some isolates in terms of infection over others reported in the past and confirmed in the current study, improve host-pathogen understanding that will benefit the development of fungal-based pathogens.

5. Conclusions

In conclusion, these findings revealed that isolate 8453 greatly disturbed the nutritional indices and host antioxidant defense, ultimately leading to the death of infected red palm weevil larvae. Significant differences in feeding performance and mortality among exposed red palm weevil larvae might considerably influence through viability and relative hydrophobicity of the spores of invading pathogens. In the future, more attention should be devoted to formulating the most virulent isolate to manage the populations of red palm weevil that damage different species of palms.

Acknowledgements

The study was partially supported by the King Abdul Aziz City for Science and Technology, Kingdom of Saudi Arabia (Project No. AT-34-293).

REFERENCES

[1] A. Hussain, M. Rizwan-ul-Haq, A.M. Al-Jabr, H.Y. Al-Ayied, Managing invasive populations of red palm weevil: A worldwide perspective, J. Food, Agric. Environ. 11 (2013) 456–463.

[2] V.A. Abraham, K.M.A. Koya, C. Kurian, Evaluation of seven insecticides for control of red palm weevil, *Rhynchophorus ferrugineus*, J. Plant. Crop. 3 (1975) 71–72.

[3] K.M. Azam, S.A. Razvi, Control of the red palm weevil, *Rhynchophorus ferrugineus* Olivier, using prophylactic spraying of date palms and trunk injection, in: 2nd Int. Conf. Date Palms, 2001: pp. 216–222.

[4] A.M. Al-Shawaf, A.A. Al-Shagagh, M.M. Al-Bakshi, S.A. Al-Saroj, S.M. Al-Badr, A.M. Al-Dandan, A. Ben Abdallah, Toxicity of some insecticides against red palm weevil *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae), Indian J. Plant Prot. 38 (2010) 13–16.

[5] A.M. Aljabr, M. Rizwan-Ul-Haq, A. Hussain, A.I. Al-Mubarak, H.Y. Al-Ayied, Establishing midgut cell culture from *Rhynchophorus ferrugineus* (Olivier) and toxicity assessment against ten different insecticides, Vitr. Cell. Dev. Biol. - Anim. 50 (2014) 296–303. doi:10.1007/s11626-013-9694-1.

[6] M. Shar, M. Rustamani, S. Nizamani, L. Bhutto, Red palm weevil (*Rhynchophorus ferrugineus* Olivier) infestation and its chemical control in Sindh province of Pakistan, African J. Agric. Res. 7 (2012) 1666–1673. doi:10.5897/AJAR11.896.

[7] T.P. Cabello, J. de la Peña, P. Barranco, J. Belda, Laboratory evaluation of imidacloprid and oxamyl against *Rhynchophorus ferrugineus*, Tests Agrochem. Cultiv. 18 (1997) 6–7.

[8] A.I. Al-Samarrie, A. Abo-Akela, Distribution of injected pesticides in date palm trees, Agric. Biol. J. North Am. 2 (2011) 1416–1426.

[9] A. Abdel-Salam, A. El-Bana, E.E. El-Rehewy, Evaluation of some insecticides on infestation of red palm weevil *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae), J. Plant Prot. Pathol. - Mansoura Univ. 5 (2014) 567–571.

[10] M. Ferry, S. Gomez, Assessment of risks and potential of injection techniques in integrated programs to eradicate the red palm weevil: Review and new perspectives, Fruits. 69 (2014) 143–157. doi:10.1051/fruits/2014005.

[11] M. Abbas, Evaluation methods for red palm weevil control in Egypt during (1992-2010), in: Palm Pest Mediterr. Conf., Nice, France, 2013. http://www.fredon-corse.com/standalone/2/ED07pqF39RpOOpN76qbZ2w1D.pdf.

[12] H. Al-Ayedh, A. Hussain, M. Rizwan-ul-Haq, A.M. Al-Jabr, Status of insecticide resistance in field-collected populations of *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae), Int. J. Agric. Biol. 18 (2016) 103–110. doi:10.17957/IJAB/15.0070.

[13] E. Llácer, C. Santiago-Álvarez, J.A. Jacas, Could sterile males be used to vector a microbiological control agent? The case of Rhynchophorus ferrugineus and Beauveria bassiana, Bull. Entomol. Res. 103 (2013) 241–250. doi:10.1017/S0007485312000582.

[14] M. Saleh, G. Hegazy, M. Salem, S. Hanounik, O. Al-Mohanna, M. Alheji, Persistence of *Steinernema carpocapsae* (Nematoda: Steinernematidae) and *Beauveria bassiana* (Deuteromycotina: Hyphomycetes) in soil around date palm trunks and their effect on adults of *Rhynchophorus ferrugineus*, Egytian J. Biol. Pest Control. 14 (2004) 141–145.

[15] R. Labbé, D. Gillespie, C. Cloutier, J. Brodeur, Compatibility of an entomopathogenic fungus with a predator and a parasitoid in the biological control of greenhouse whitefly, Biocontrol Sci. Technol. 19 (2009) 429–446.doi:10.1080/09583150902803229.

[16] A. Hussain, S. Ahmed, M. Shahid, Laboratory and field evaluation of *Metarhizium anisopliae* var. *anisopliae* for controlling subterranean termites, Neotrop. Entomol. 40 (2011) 244–250.

[17] E.D. Quintela, G.M. Mascarin, R.A. da Silva, J.A.F. Barrigossi, J.F. da S. Martins, Enhanced susceptibility of *Tibraca limbativentris* (Heteroptera: Pentatomidae) to *Metarhizium anisopliae* with sublethal doses of chemical insecticides, Biol. Control. 66 (2013) 56–64. doi:10.1016/j.biocontrol.2013.03.018.

[18] M. Ahmad Malik, M. Manzoor, H. Ali, A. Muhammad, S. ul Islam, M. Qasim, N. Ahmad, A. Idrees, A. Muhammad, H. Sohaib Ahmed Saqib, C. Habib Ali, Evaluation of imidacloprid and entomopathogenic fungi, *Beauveria bassiana* against the red palm weevil *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae), J. Entomol. Zool. Stud. JEZS. 262 (2016) 262–268.

[19] W. Akbar, J.C. Lord, J.R. Nechols, T.M. Loughin, Efficacy of *Beauveria bassiana* for red flour beetle when applied with plant essential oils or in mineral oil and organosilicone carriers, J. Econ. Entomol. 98 (2005) 683–688. doi:10.1603/0022-0493-98.3.683.

[20] C. Murali Mohan, K.A. Lakshmi, K.U. DEVI, Laboratory evaluation of the pathogenicity of three isolates of the entomopathogenic fungus *Beauveria bassiana* (Bals.) Vuillemin on the American cockroach (*Periplaneta americana*), Biocontrol Sci. Technol. 9 (1999) 29–33. doi:10.1080/09583159929884.

[21] L.L. Mnyone, M.J. Kirby, D.W. Lwetoijera, M.W. Mpingwa, E.T. Simfukwe, B.G. Knols, W. Takken, T.L. Russell, Tools for delivering entomopathogenic fungi to malaria mosquitoes: Effects of delivery surfaces on fungal efficacy and persistence, Malar. J. 9 (2010) 246. doi:10.1186/1475-2875-9-246.

[22] A. Hussain, M. Rizwan-ul-Haq, H. Al-Ayedh, A. AlJabr, Susceptibility and immune defence mechanisms of *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae) against entomopathogenic fungal infections, Int. J. Mol. Sci. 17 (2016) 1518. doi:10.3390/ijms17091518.

[23] A. Hussain, M. Rizwan-ul-Haq, H. Al-Ayedh, S. Ahmed, A.M. Al-Jabr, Effect of *Beauveria bassiana* infection on the feeding performance and antioxidant defence of red palm weevil, *Rhynchophorus ferrugineus*, BioControl. 60 (2015) 849–859. doi:10.1007/s10526-015-9682-3.

[24] L. Lacey, T. Liu, J. Buchman, J. Munyaneza, J. Goolsby, D. Horton, Entomopathogenic fungi (Hypocreales) for control of potato psyllid, *Bactericera cockerelli* (Šulc) (Hemiptera: Triozidae) in an area endemic for zebra chip disease of potato, Biol. Control. 56 (2011) 271–278. doi:10.1016/j.biocontrol.2010.11.012.

[25] M. Feng, J. Johnson, S. Halbert, Natural control of cereal aphids (Homoptera: Aphididae) by entomopathogenic fungi (Zygomycetes: Entomophthorales) and parasitoids (Hymenoptera: Braconidae and Encyrtidae) on irrigated spring wheat in Southwestern Idaho, Environ. Entomol. 20 (1991) 1699–1710. doi:10.1093/ee/20.6.1699.

[26] R.J. Harris, S.J. Harcourt, T.R. Glare, E.A.F. Rose, T.J. Nelson, Susceptibility of *Vespula vulgaris* (Hymenoptera: Vespidae) to generalist entomopathogenic fungi and their potential for wasp control, J. Invertebr. Pathol. 75 (2000) 251–258. doi:10.1006/jipa.2000.4928.

[27] A. Hussain, Y.F. Li, Y. Cheng, Y. Liu, C.C. Chen, S.Y. Wen, Immune-related transcriptome of *Coptotermes formosanus* Shiraki workers: The defense mechanism, PLoS One. 8 (2013) e69543. doi:10.1371/journal.pone.0069543.

[28] A. Hussain, M.Y. Tian, Y.R. He, J.M. Bland, W.X. Gu, Behavioral and electrophysiological responses of *Coptotermes formosanus* Shiraki towards entomopathogenic fungal volatiles, Biol. Control. 55 (2010) 166–173. doi:10.1016/j.biocontrol.2010.08.009.

[29] A. Hussain, M.Y. Tian, Y.R. He, S. Ahmed, Entomopathogenic fungi disturbed the larval growth and feeding performance of *Ocinara varians* (Lepidoptera: Bombycidae) larvae, Insect Sci. 16 (2009) 511–517. doi:10.1111/j.1744-7917.2009.01272.x.

[30] G. Inglis, D. Johnson, M. Goettel, Effects of temperature and sunlight on mycosis (*Beauveria bassiana*) (Hyphomycetes: Sympodulosporae) of Grasshoppers under field conditions, Environ. Entomol. 26 (1997) 400–409. doi:10.1093/ee/26.2.400.

[31] B.C. Murphy, T.A. Morisawa, J.P. Newman, S.A. Tjosvold, M.P. Parrella, Fungal pathogen controls thrips in greenhouse flowers, Calif. Agric. 52 (1998) 32–36. doi:10.3733/ca.v052n03p32.

[32] A. Hussain, M. Rizwan-ul-Haq, H. Al-Ayedh, A. Al-Jabr, Mycoinsecticides: Potential and future perspective, Recent Pat. Food. Nutr. Agric. 6 (2014) 45–53. doi:10.2174/2212798406666140613113905.

[33] Ó. Dembilio, E. Quesada-Moraga, C. Santiago-Álvarez, J.A. Jacas, Potential of an indigenous strain of the entomopathogenic fungus *Beauveria bassiana* as a biological control agent against the red palm weevil, *Rhynchophorus ferrugineus*, J. Invertebr. Pathol. 104 (2010) 214–221. doi:10.1016/j.jip.2010.04.006.

[34] M.J. Hajjar, A.M. Ajlan, M.H. Al-Ahmad, New approach of Beauveria bassiana to control the red palm weevil (Coleoptera: Curculionidae) by trapping technique, J. Econ. Entomol. 108 (2015) 425–432. doi:10.1093/jee/tou055.

[35] R. El-Sufty, S. Al-Bgham, S. Al-Awash, A. Shahdad, A. Al-Bathra, A Trap for auto-dissemination of the entomopathogenic fungus *Beauveria bassiana* by red palm weevil adults in date palm plantations, 21 (2011) 271–276.

[36] R. El-Sufty, S. Al-Awash, A. Al-Amiri, A. Shahdad, Biological control of red palm weevil , *Rhynchophorus ferrugineus* (Col .: Curculionidae) by the entomopathogenic fungus *Beauveria bassiana* in United Arab Emirates, Biol. Control. (2007) 399–404.

[37] G. Gindin, S. Levski, I. Glazer, V. Soroker, Evaluation of the Entomopathogenic Fungi *Metarhizium anisopliae* and *Beauveria bassiana* against the Red Palm Weevil *Rhynchophorus ferrugineus*, Phytoparasitica. 34 (2006) 370–379.

[38] A. Hussain, M.Y. Tian, Y.R. He, L. Ruan, S. Ahmed, *In vitro* and *in vivo* culturing impacts on the virulence characteristics of serially passed entomopathogenic fungi, J. Food, Agric. Environ. 8 (2010) 481–487.

[39] SAS Institute, SAS user's guide: Statistics. SAS Institute, Cary, (2000).

[40] H. Girardin, S. Paris, J. Rault, M.N. Bellon-Fontaine, J.P. Latgé, The role of the rodlet structure on the physicochemical properties of *Aspergillus* conidia, Lett. Appl. Microbiol. 29 (1999) 364–369. http://www.ncbi.nlm.nih.gov/pubmed/10664979.

[41] W.S. Abbott, A method of computing the effectiveness of an insecticide, J. Econ. Entomol. 18 (1925) 265–267. doi:10.1093/jee/18.2.265a.

[42] Statistix, Statistix 8.1 Tallahassee, FL: Analytical Software, (2003).

[43] R.M. Russell, J.L. Robertson, N.E. Savin, POLO: A new computer program for Probit analysis, Bull. Entomol. Soc. Am. 23 (1977) 209–213. doi:10.1093/besa/23.3.209.

[44] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ Method., Methods. 25 (2001) 402–408. doi:10.1006/meth.2001.1262.

[45] Z.L. Wang, S.H. Ying, M.G. Feng, Recognition of a core fragment of *Beauveria bassiana* hydrophobin gene promoter (P *hyd1*) and its special use in improving fungal biocontrol potential, Microb. Biotechnol. 6 (2013) 27–35. doi:10.1111/j.1751-7915.2012.00351.x.

[46] S. Ahmed, M.R. Ashraf, M.A. Hussain, M.A. Riaz, Pathogenicity of isolates of *Metarhizium anisopliae* from Murree (Pakistan) against *Coptotermes heimi* (Wasmann) (Isoptera: Rhinotermitidae) in the laboratory, Pakistan Entomol. 53 (2013) 1689–1699. doi:10.1017/CBO9781107415324.004.

[47] L.B. Silva, W. Silva, M.L.R. Macedo, M.T.L.P. Peres, Effects of *Croton urucurana* extracts and crude resin on *Anagasta kuehniella* (Lepidoptera: Pyralidae), Brazilian Arch. Biol. Technol. 52 (2009) 653–664. doi:10.1590/S1516-89132009000300018.

[48] A. AlJabr, A. Hussain, M. Rizwan-ul-Haq, H. Al-Ayedh, Toxicity of Plant Secondary Metabolites Modulating Detoxification Genes Expression for Natural Red Palm Weevil Pesticide Development, Molecules. 22 (2017) 169. doi:10.3390/molecules22010169.

[49] A. Hussain, M. Rizwan-ul-Haq, H. Al-Ayedh, A.M. Aljabr, Toxicity and detoxification mechanism of black pepper and its major constituent in controlling *Rhynchophorus ferrugineus* Olivier (Curculionidae: Coleoptera), Neotrop. Entomol. (2017). doi:10.1007/s13744-017-0501-7.