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Isolation, phenotypic and genotypic characterization of indigenous *Beauveria bassiana* isolates from date palm infested with *Rhynchophorus ferrugineus* in Hail region, Saudi Arabia

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ABSTRACT

The red palm weevil (RPW) *Rhynchophorus ferrugineus* overrun date palm ranches in many parts of Saudi Arabia, henceforth causes massive economic losses. Integrated pest Management (IPM) of the RPW by utilizing Entomopathogenic fungi (*Beauveria bassiana*), which has antagonistic activity against many insect pests, was the main objective of the current study. In the present study, soil samples, samples of dead red palm weevils (RPW) and palm fronds were collected according to RPW incidence map of Hail region, Saudi Arabia. Isolates of entomopathogenic fungi were isolated from the dead RPW adults and larvae. The fungal culture (BSA1, BH-2 and BH-3) was preserved and maintained for further analysis. Morphological and biochemical characterization of the antagonist fungi were employed and confirmed that the fungus belonged to *Beauveria* spp. Further, this fungal isolates were propagated and prepared for genetic characterization. Sequencing of internal transcribed spacers (ITS1 and ITS2) region was shown that three polymorphic ITS regions. The molecular identification of the fungus strain was employed at King Faisal University-the College of Agriculture and Food Science and confirmed that the fungus identified as *Beauveria bassiana* as the first record of this beneficial species in Hail region.

KEY WORDS: DATE PALM, RED PALM WEEVIL, FUNGUS, MORPHOLOGICAL, BIOCHEMICAL, MOLECULAR IDENTIFICATION

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INTRODUCTION

The red palm weevil (RPW) *Rhynchophorus ferrugineus* Olivier (Coleoptera: Curculionidae), is an overwhelming palm pest that can cause tremendous financial losses worldwide throughout the previous 30 years, as they are notable to assault several species (more than 200) of palm including the date palm (*Phoenix dactylifera* L.) (Murphy and Briscoe, 1999; Barranco *et al.*, 2000; Faleiro, 2006). These large economic losses in date palms could ascribed to the way that, to-date, there are no powerful control measures. *Rhynchophorus ferrugineus* is broadly geographically distributed in Africa, Asia, Europe, Oceania, and North America (EPPO, 2006; 2007a; 2007b; 2009; Azmi *et al.,* 2017).

The entomopathogenic fungus *Beauveria bassiana* (*Ascomycota*, *Hypocreales*) has demonstrated use within insect biocontrol administrations for concealment of numerous crop. It infects a an extensive variety of insect pests of socioeconomic importance pests (Bing & Lewis 1991, 1992, Krueger & Roberts 1997; Mulock & Chandler 2000). Recently, *B. bassiana,* was additionally found to occur naturally as an endophyte in plant tissues, for example, as leaves, twigs, wood and bark in specific plants like maize, cotton, wild cacao, white pine, coffee and furthermore been set up as an endophyte artificially in specific crops like, maize, cotton, tomato, opium poppy, cacao, coffee, date palm, banana, sorghum and jute. The entomopathogenic fungus *B. bassiana* isolated from dead *R. ferrugineus* cadavers gave more mortality compared to the other isolates. In the virulence bioassay two isolates of *B. bassiana* shown the highest percentage of larval and adult mortality at all exposure which recommend that they may be the most effective isolates for sustainable insect control programs, (Yasin *et al.*,2017).

Endophytes have several advantages; firstly, they are inside the plant tissues and constantly shielded from abiotic push factors. Also, the application cost is less because of limited application through seed treatment or seedling dip or foliar spray. Moreover, once settled as an endophyte, they may offer season-long protection against the pests that have secretive life cycle by causing encouraging discouragement or antibiosis (Vega *et al.*, 2008) or prompting their mortality or less pervasion. All these properties of fungal endophytes make them reasonable to be utilized as a bio-control agent to protect crops from the pests. Insect pests such as the European corn borer (*Ostrinia nubilalis*) in U.S.A., and the banana weevil (*Cosmopolites sordidus*) in Uganda respectively were effectively controlled by endophytic establishment of *B. bassiana*.

Date palms are considered as the image of life in the leave, since it endures high temperatures, and saltiness when contrasted with numerous other fruit crop species. One of the most established relationships that man has had with a tree has been with date palms, which have been developed since ancient times (Zohary and Hopf, 2012). One of the character of date palm is that it can adapt to extreme drought, to heat, and to relatively high levels of soil salinity. Nevertheless, extreme quantities of salinity due to irrigation with saline water result to a significant decrease in the productivity of the fruits. It is important to study the mechanism of tolerance to these abiotic stress in order to develop future date palm varieties that can tolerate excessive soil salinity (Yaish & Kumar 2015).

Several insects invade date palm trees, of which the red palm weevil (RPW), *R. ferrugineus* (Olivier) (Coleoptera: Curculionidae), is a standout amongst the most essential and harming pests, being a noteworthy risk to date palm trees everywhere throughout the world. The red palm weevil is ordinarily well hidden, and numerous local pervasions have just recently been perceive, making the red palm weevil a pest of major economic significance in all Persian Gulf Countries. This study aimed to isolation and screening of antagonistic *B. bassiana* from diverse soils and date palm and plantation crop ecosystem in Hail region, Saudi Arabia.

MATERIAL AND METHODS

Soil samples, samples of infected and dead red palm weevil (RPW) insects and date plant materials collected according to RPW incidence map of Hail, Saudi Arabia during the period 2016-2017. The *B. bassiana* (BH-2) strain was isolated from soil sample collected from Al Koutha village in Hail region. About 500 grams of soil sample were placed in a plastic bag. Five larvae (L3-L4) of the red palm weevil *R. ferrugineus* were added to the soil substrate to be infected by the entomopathogenic fungi. The plastic bag was kept for 2-3 weeks at room temperature (20-25°C) and a ca. 80% humidity level was ensured inside the bag by periodic water sprays. Dead larvae were collected, placed on wet filter paper in Petri dishes and put in an incubator at 25°C. The mycelium and spores that emerged on larvae were transferred directly onto growth medium (PDA – PotatoDextrose Agar) in Petri dishes and incubated at 25°C for 10 days; the isolates were then purified by repeated transplanting (Goettel and Inglis, 1997).

The *B. bassiana* (BH-1, BH-2 and VBM) isolates were obtained from naturally infected RPW adults collected in from Jubbah village in Hail region (Fig 1). To promote conidial growth, mycosed RPW adult cadavers were placed separately on filter paper soaked daily with water to achieve ca.100% RH inside Petri dishes. The petri dishes were incubated at room temperature (20-25C°).

FIGURE 1. Fungal growth of red palm weevil in Hail region.

Parts of fungal propagules grown on the cadavers were then transferred, with sterile needles, into Petri dishes with SDAY1/4 (Sabouraud Dextrose Agar Fluka supplemented with yeast extract ¼of concentration) and kept in an incubator at 25°C. Pure fungal colonies were then stored on PDA (Potato Dextrose Agar) and MEA (Malt Extract Agar) slants in bacteriological glass tubes at 4°C. The *B. bassiana* isolates were confirmed by sequencing analyses of the 18SrRNA gene and the internal transcribed spacer (ITS1).

For microscopic identification of the fungus, pure fungal culture was used after successive purification. With the help of inoculating needle some portion of growth of the fungus was teased and place it on the slide then, 70% ethanol was used for washing, then ethanol was removed by blotting paper. Then, a drop of Lacto-phenol cotton blue was kept, and the mycelium was spread with needles, and a cover slip placed and examined under microscope with high power objective. The morphology and spore structures were noted.

API 20 C AUX kit was used to identify the strains of yeast isolated from RPW samples according to the manufacturer's instructions. Yeast strains were purified by culturing them on PDA (Potato Dextrose Agar) medium and incubated for 72 hours at 25°C. The strains were purified twice before identification. After 48 and 72 hours the growth was compared in each cupule to the 0 cupule, which is used as a negative control. A cupule more turbid than the control indicates a positive reaction to be recorded in the result sheet. Each strain was identified with the identification software by manually entering the 7-digit numerical profile via the keyboard.

The molecular analyses for the fungal isolates were performed at King Faisal University- the College of Agriculture and Food Science – Pest & Plant Disease Unit.

All isolates were re-grown on other PDA petri dishes and inoculated on Potato dextrose broth medium for microscopic examination (Fig 2), also for extraction of DNA and molecular identification.

DNA was extracted using modified method of Dellaporta and Hicks, 1983 as the following protocol: Twenty mgs of frozen- dried mycelium or fresh harvested mycelium were ground with Kontes pestles in a 1.5 ml tube with 500 µl of Dellaporta buffer (100 mMTris pH 8. 50 mMethylenediamine-tetraacetate EDTA, 500 mMNaCl, 10 mM beta mercaptoethanol (BME). Thirty three µl of 20% sodium dodecyl sulfate (SDS, w/v) were added, and incubate the mixture was vortexed and incubated for 10 min at 65°C.160µl of 5 M potassium acetate KoAc (Sigma chemicals) were added and vortexed. The mixture was spun for 10 min at 10,000 rpm in a microcentrifuge tube. 450 µl of supernatant were transferred to a new tube. 450 µl phenol, chloroform and isoamylalcohol (PCI) 25:24:1 were added and vortexed for 5min and then spun for 5 min at 10,000 rpm. 400 µl of the upper phase were removed to a clean micro-centrifuge tube and 0.5 volumes of isopropanol were added, vortexed and spinet for 10 min at 14,000 rpm. The supernatant was removed, the total nucleic acid was precipitated in the bottom of the tube. The pellet was washed with 70% ethanol and spun 5 min at 10,000 rpm. The pellet was resuspended in 100 µl of ddH2O.

Two primer pairs, the forward IT5 primer 5'-GGAAGTAAAAGTCGTAACAAGG-3') and the reverse ITS4 primer (5'-TCCTCCGCTTATTGATATGC-3') were used to

FIGURE 2. Plates shown *Beauveria bassianaafter* re-grown in PDA in PPDUlab (A,B). C and D *B. bassianaas* received form University of Hail.

amplify the entire ITS region (White *et al.*, 1990). PCR was done in a 25 µl reaction containing 1 µl of the fungal DNA extract (40 ng of total DNA), 2 mM MgCl2, 2.5 of 10x PCR buffer,1.5 µL of 10 µM of each primer, 2.5 µl of 10 mMdNTPs, 0.3 µl of 5U Taq DNA Polymerase and the reaction was completed to 25L with Nuclease-free water. PCR was conducted in the ESCO Swift Maxi Thermal Cycler with initial denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 30 sec, 52°C for 30 sec, and 72°C for 30 sec, and the final cycle is a polymerization cycle performed at 72°C for 10 min. PCR Products were purified using QIAquick® PCR Purification Kit (Cat. No. 28106) according to manufacturing procedures.

The purified PCR products were sequenced by Macrogen Inc., (Korea), and sequencing of the purified isolates was performed in both directions using ITS5 and ITS4 primer pairs. Sequence alignments were edited by MEGA6 (Tamura et al., 2013).

RESULTS AND DISCUSSION

Based on the API 20 AUX test performed on selected isolates of *Beauveria* spp. (Table 1), *Beauveria basiana* was the predominant yeast strain in all samples, also there was small percentage of *Aspergillus* spp. On the basis of the API 20 AUX test, the isolated yeast was identified as *Beauveria basiana*. Its profile makes up to 90% of the strains. As shown in the table, various assimilation profiles were obtained for *Beauveria basiana*. Studies on assimilation profiles were based on the acidification of

Table 1. The biochemical profile of *Beauveria* spp.

Sugar	Reaction	Sugar	Reaction	Sugar	Reaction
esculin	-	Adonitol	+	salicin	+
D-arabinose	-	rhamnose	+	glycogen	-
dulcitol	-	Inositol	+	dextrose	+/-
D-xylose	-	Lactose	+	trehalose	+
raffinose	-	sorbitol	+	maltose	+
galactose	+	dextrine	+/-	sucrose	+
D-fructose	+	mannitol	-	dolicitol	-

Note: + = Positive reaction
+/- = Weak reaction
- = Negative reaction

twenty sugars. The results indicate that galactose, fructose, rhamnose, maltose, sucrose and trehalose were assimilated at high degree contrasted with esculin, arabinose, xylose, raffinose, mannitol, glycogen and dulcitol. Biochemical properties and specifically their use of sugars can be utilized to help the morphology and are valuable for recognizing species of *Beauveria*. Mugnai *et. al.* (1989) contemplated the intra- and interspecific variety of 32 isolates appointed to the genus *Beauveria*. They presumed that cultural characters were profoundly factor also, couldn't be utilized dependably to separate species.

Depending on cultural and microscopic examination, fungal isolates belonged to two genera *Beauveria* and *Aspergillus* as shown on Table (2).

Table 2. Fungal speices isolated from *R. ferrugineus* in Hail region.

No	Label	Colour	Fungus name
1	BSA-1	white	Beauveria bassiana
2	BH-1	brown	Aspergillus ssp.
3	BH-2	white	Beauveria bassiana
4	BH-3	white	Beauveria bassiana
5	VBM	green	Aspergillus ssp.

All extracted DNAs from the three fungal isolates gave a clear bands on the expected size ≈ 600 bp using primer pairs ITS5&ITS4 (Fig 3). Blast analysis revealed that the fungal isolates are *Beauveria bassiana*. Sequences alignment (Fig 3) showed that all isolates had same sequence with 100% similarity with chinses isolates (Accession No. JQ320361). All sequences of the three isolates were deposited in the Genbank. 16 S rDNA nucleotide sequence has been sent to the Genbank for sequence publication.

The red palm weevil *R. ferrugineus* is a standout amongst the most serious pests of various date palm species, including date palms (Giblin-Davis, 2001). The weevils create inside the tree trunk, wrecking its vascular system and in the long run causing the crumple and death of the tree. The pest is generally circulated in Oceania, Asia, Africa and Europe. The RPW makes extreme harm to coconuts in Southeast Asia (Giblin-Davis, 2001). It showed up in the Middle East in the 1980s and has vigorously harmed date production by pulverizing huge number of date palms (Murphy and Briscoe 1999). Invasion was first reported in Israel and Jordan in 1999 (Khan and Gangapersad 2001).

The enormous economic losses created by RPW for date palm trees in Hail region encouraged the authors

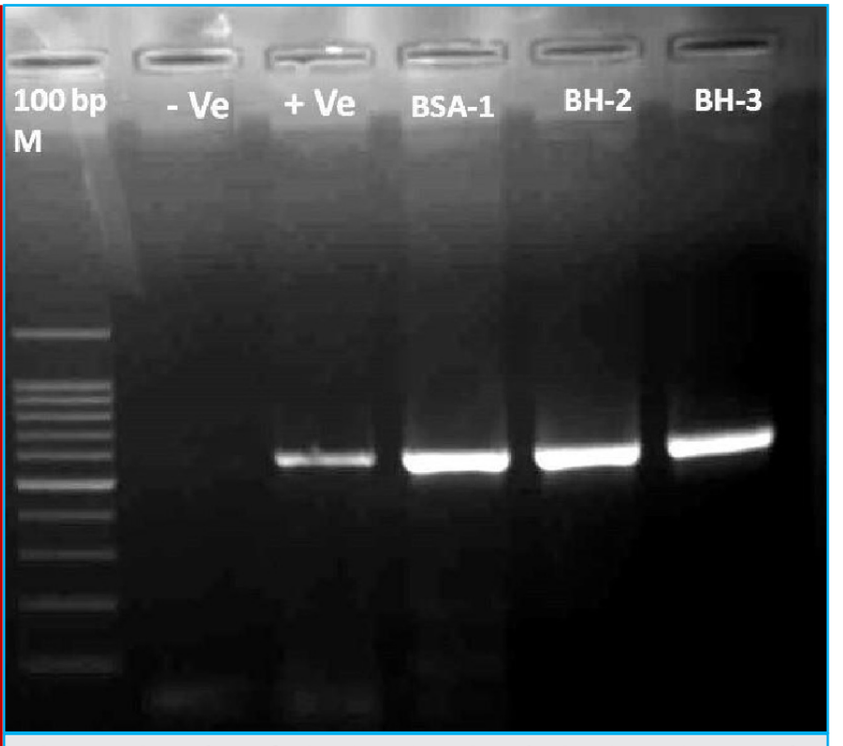

FIGURE 3. PCR products of ITS region using ITS5 and ITS4 primer pairs. M: 100 bp DNA Ladder; -Ve is the non-template control while +Ve is the positive control, BSA-1, BH-2 and BH-3 are *Beauveriabassiana* isolates from Hail.

to seek for an economically feasible technique to be employed for eradicating this harmful insect. During surveying the various infected date palm farms, many dead RPW were found under the infected trees covered with whitish material. In addition, farmers informed the researchers that they do not use any chemical treatment for pest control in their farms. This information motivated the researchers to try to recognize the causes of RPW death. Hence, the research has been resumed by collecting the dead insects are identifying the whitish material covering their bodies. The morphological tests indicated that the whitish materials were fungus growth. Subsequently, the biochemical identification tests indicated that the fungus was *Beauveria bassiana*. Then, the identification was confirmed using molecular biology tests.

Fungi are the commonest reason of insect disease in nature. Certain species of entomopathogenic fungi shown specificity to certain host (Wang, Chengshu & Wang, Sibao. 2017). Fungi may infect insects by direct infiltration of the cuticle thus works as contact insecticides. *Beauveria* is standout amongst other known genera of entomopathogenic fungi and worldwide various enrolled mycoinsecticide formulations in view *Beauveria bassiana* is utilized for control of insect pests (Thomas and Read, 2007). This fungus has an especially wide host range (over 700 species) enabling it to be utilized against vectors of human disease and an extensive variety of insect pests (de Faria, and Wraight, 2007). For instance in China, around one million hectares a year are treated with *Beauveria bassiana* to control forest insects such as the pine caterpillar *Dendrolimus punctatus* (Wang *et al.,* 2004)

Beauveria is outstanding for creating huge cluster of biological active secondary metabolites including non-peptide pigments and polyketides (e.g., *oosporein, bassianin* and *tenellin*), nonribosomally synthesized peptides (e.g., *beauvericin, bassianolides* and *beauveriolides*), and discharged metabolites associated with pathogenesis and destructiveness (e.g., oxalic acid) that have potential or acknowledged modern, pharmaceutical and agricultural uses (Xu *et al*., 2009). The mechanism of action and biological function of Oosporein have persisted unclear. *B. bassiana* has developed the ability to parasitize insects. A unique zinc finger transcription factor, BbSmr1 (*B. bassiana* secondary metabolite regulator 1), was identified in a screen for oosporein overproduction. Deletion of *Bbsmr1* resulted in up-regulation of the oosporein biosynthetic gene cluster (*OpS* genes) and constitutive oosporein production, (Fan *et al*., 2017).

The dominant part of endophyte explore has concentrated to date on the vertically-transmitted endophytes inside the genus *Neotyphodium* (Clavicipitaceae) that systemically colonize the over the ground parts of a few grasses. These clavicipita-ceous endophytes are generally known to present a variety of potential advantages to their grass host plants (Kuldau and Bacon, 2008). Less consideration has been given to the evenly transmitted non-clavicipitaceous endophytes, which are in nature and commanded by the Ascomycetes (Arnold and Lutzoni, 2007); of which a few genera are fungal entomopathogens (Ascomycota: Hypocreales).

Rising as an energizing new area of research, 'fungal ento-mopathogens as endophytes' has just rather recently been incorporated into a more than 100 old endophyte research base after the recuperation of different genera of fungal ento-mopathogens as endophytes from various plant species (Vega et al., 2008). Some of these fungi have been accounted for as normally happening endophytes, while others have been brought into the plant utilizing distinctive different inoculation techniques. Spearheading take a shot at entomopathogenic endophytes was directed with *B. bassiana* (Balsamo), a universal soil-borne fungus that infects an extensive variety of various insects (>700 insect species; Inglis *et al.*, 2001) and is a standout amongst the most marketed fungal biopesticides (de Faria and Wraight, 2007).

Lewis and Cossentine (1986) credited the season-long concealment of the European corn borer *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae) in maize *Zea mays* L. (Poaceae), estimated as reduced tunneling by the insect, to the establishment of *B. bassiana* as an endophyte following utilization of a watery suspension of the fungus to the plants. Ensuing work by Lewis and colleagues utilizing a similar model system demonstrated fruitful re-isolation of *B. bassiana* from interior plant tissues after utilization of the fungus utilizing different inoculation methods. These antagonist funfi can have beneficial effects on host plants, e.g., plant growth promotion, reducing disease severity, inducing plant defense mechanisms, and producing anti-herbivore products (Arnold and Lewis, 2005, Amatuzzi, *et al*., 2017).

In addition to maize, a wide assortment of host plants (counting both agronomic and weed species) have additionally been appeared to harbor *B. bassiana* as an endophyte. As opposed to *B. bassiana*, the host plant scope of other fungal entomopathogens is as yet developing. For example, *Verticillium (=Lecanicillium) lecanii* (Zimm.) Viegas has been accounted for as a natural endophyte in bear-berry *Arctostaphylos uva-ursi* L. (Ericaceae) (Widler and Muller, 1984) and ironwood (Bills and Polishook, 1991). A relatively recent development, the use of some fungal entomopathogens could function as biofertilizers. Various inoculation techniques (e.g., foliar sprays, soil drenching, seed soaking, injections, etc.) are effective in introducing fungal entomopathogens as

endophytes, but colonization appears to be localized and ephemeral, (Vega 2018).

There is presently considerable confirmation that some endophytic fungal entomopathogens, especially *B. bassiana* and *Lecanicillium* spp. (some time ago *Verticillium lecanii*), may likewise exhibit hostile action against plant pathogens , in addition to their outstanding biocontrol activity against insect pests. This proposes that these entomopathogens have a promising potential to be developed as biopesticides for numerous reasons in IPM methodologies (Goettel *et al*., 2008; Vega *et al.,* 2009; Ownley *et al*., 2010). *Beauveria bassiana* strain 11–98, applied as a seed treatment, has been accounted to suppress damping-off caused by the soil-borne pathogens, *Rhizoc-tonia solani* Kuhn (Basidiomycota: Cantharellales) and *Pythium myriotylum* Drechsler (Oomycota: Pythiales), in tomato (Ownley *et al*., 2004; Clark *et al*., 2006) and cotton seedlings (Griffin, 2007; Ownley *et al*., 2008). Pre-treatment of cotton seedlings with the same *B. bassiana* strain likewise resulted in reduced seriousness of bacterial blight caused by *Xanthomonas axonopodis* pv. malvacearum (Xam) (Griffin *et al*., 2006; Ownley *et al*., 2008).

More recently, several strains of *B. bassiana* were found to fundamentally diminish the rate and seriousness of the Zucchini yellow mosaic virus (ZYMV; genus Potyvirus, family Potyviridae) in squash (Jaber and Salem, 2014) and downy mildew caused by *Plasmopara viticola* (Berk. and Curt.) Berl. and de Toni. (Oomycota: Peronosporaceae) in grapevines (Jaber, 2015) following foliar inoculation of plants with conidial suspensions of the tested strains.

CONCLUSION

Strains of *Beauveria bassiana* (Bals.-Criv.) Vuill. and *Metarhizium anisopliae* (Metchn.) Sorokin (Hypocreales: Clavicipitaceae) have been isolated from wild *R. ferrugineus* populations (Lo Verde et al., 2015). *Beaveria bassiana* fungus could be used as one of the biological strategies in controlling red palm weevil (Hajjar *et. al*., 2014). Based on the morphological, biochemical and molecular biological techniques, *B. bassiana* was isolated and identified as the fungal strain for the first time in Hail region, Saudi Arabia in which this fungal strain can be utilized in the biological control of the red palm weevil *R. ferrugineus* which is considered as a destructive pest of date palms in this region.

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