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Innovative Method to Control Dubas Bug, *Ommatissus lybicus* (Deberg) (Homoptera: Tropiduchidae) in Date Palm Orchards Using Endophytic *Beauveria bassiana* Isolates

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Abstract: The main objective of this study was to investigate the presence of natural endophytic *Beauveria bassiana* within date palm tissues using molecular technique and measure their field efficacies in controlling Dubas bug, *Ommatissus lybicus* (Deberg). Two entomopathogenic *B. bassiana* isolates (MARD 108 and 100) were isolated from date palm, *Phoenix dactylifera* L. leaves; in addition, one isolate (MARD 92) originally from soil was identified to have endophytic property. Concentration of 1×10^9 conidia/mL of each of three endophytic isolates was used in field experiments targeting Dubas bug nymphs via injection tree trunks. The results indicated that the high mortality rates reached 92%, 96% and 100% with infliction of the three endophytic isolates after 15 d from the treatment. The successful establishment of the fungal isolates in the date palm tissue was determined using *B. bassiana* species-specific primer for the first time via using conventional polymerase chain reaction (PCR) amplification technique before and after injection, and the positive gel band representation was the identification signs. The novel results depicted for the first time the presence of natural endophytic *B. bassiana* isolates within date palm tissues and their field efficacies in controlling Dubas bug, *O. lybicus* (Deberg) infestation.

Key words: Dubas bug, *Ommatissus lybicus*, *Beauveria bassiana*, endophytes, date palm orchards.

1. Introduction

Beauveria bassiana is worldwide known as an entomopathgenic fungus to be used to control many insect pests [1, 2]. In addition, since 1990s of the last century, *B. bassiana* was reported to be isolated from many plants as an endophyte, including maize, coffee, sorghum, banana tissue cultures, *Theobroma gileri*, *Carpinus caroliniana*, seeds and needles of *Pinus monticola*, *Opium poppies*, potato, cotton, cocklebur and jimsonweed [3-12]. The endophyte *B. bassiana* isolates were applied to manage many insect pests, such as European corn borer, *Ostrinia nubilalis*, stem borers, specifically *Chilo partellus*, *Busseola fusca* and *Sesamia calamistis*, red palm weevil, *Rhynchophorus ferrugineus* [11, 13, 14].

Date palms, *Phoenix dactylifera* L. are most economically important fruit trees in tropical and subtropical areas, and they are growing in large area in many countries including Iraq. More recently, Iraq ranked as the 7th among the countries that produce date fruit in the world [15]. Total of date palm production in Iraq are 507,000 tons from 11.9 million trees at 2009 [16]. Study is aiming to increase its production of date in the next 10 years.

Dubas bug, *Ommatissus lybicus* (Deberg) Aschae and Wilson (Homoptera: Tropiduchidae) is considered as an important pest of date palm in many countries and regions, including Iraq [17]. It was ranked as a major pest on date palm causing high yield losses

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reaching around 50% of crop production under sever infestation [18-20]. Dubas nymph and adults can damage date palm trees via sucking the sap from leaflets, midrib of frond and fruit stalks [21].

The main objective of this study was to investigate the presence of natural endophytic *B. bassiana* within date palm tissues using molecular technique and measure their field efficacies in controlling Dubas bug, *O. lybicus*.

2. Materials and Methods

2.1 Isolating Natural Endophyte from Date Palm Leaves

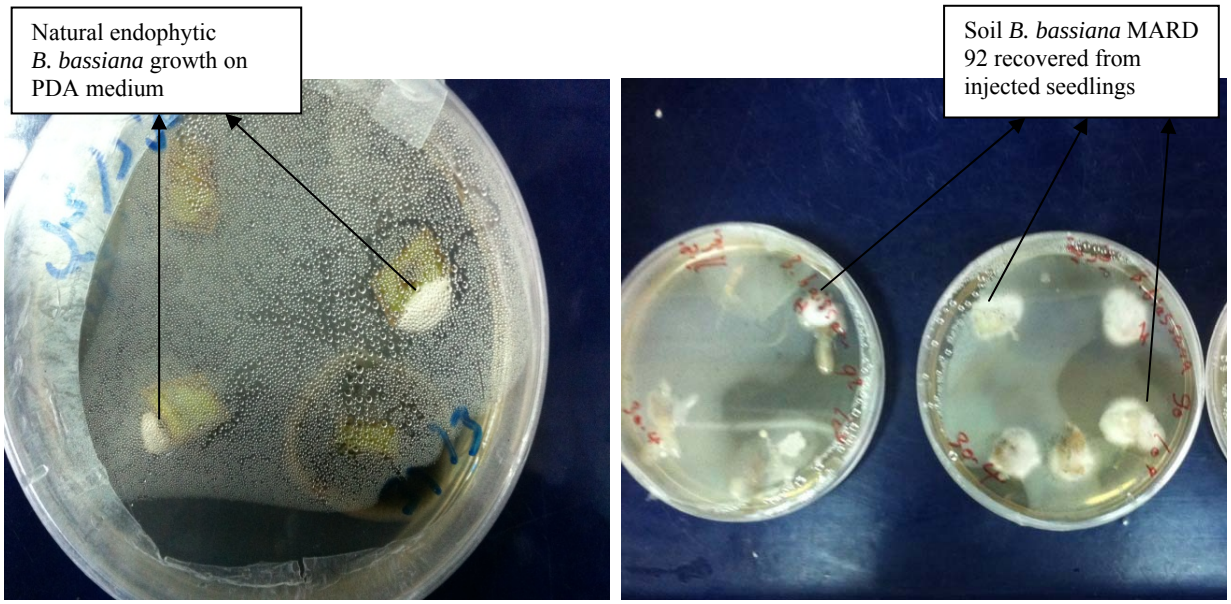
40 date palm fronds were randomly collected from date palm orchards in Al-Mada'in district (longitude 44°56′ E, latitude 33°15′ N and altitude 15.14 m), southeast of Baghdad province. Three (2-4 mm) leaf tissue pieces from each collected samples were surface sterilized with bleach (1% available chlorine) for 5 min, and washed twice in sterile water for 5 min [22]. Then, pieces were dried by placing them on sterile paper towel. Subsequently, leaf tissue pieces were transferred onto quarter-strength potato dextrose agar (PDA) plates, which contain 100 μg/mL streptomycin sulphate and 10 μg/mL tetracycline hydrochloride. Plates were incubated in the incubator with 25 °C and 70% relative humidity (RH) in the dark from 3 d to 5 d [22] before screening for any possible fungal growth (Fig. 1a).

2.2 Isolating Natural Entomopathogenic Fungi from Soil

Entomopathogenic fungi isolates MARD 24, 22, 83, 3, 54, 32, 56, 90, 91 and 92 (Fig. 1b) were screened for their enophyte ability after its injection through date palm seedlings. These were isolated using bait trap method according to Zimmermann [23], in which wax moth, *Galleria mellonella* L. larvae (Lepidoptera: Pyralidae) reared as a lab colony at 25 ± 2 °C in a rearing room were incubated with soil. Dead larvae having fungal growth were incubated on PDA media [24].

2.3 Endophyte Isolates Purification

The inoculums obtained from samples driven from natural endophyte fungal from orchards of date palm trees and natural entomopathogenic fungi from soil that found to have endophyte ability were transferred



(a) (b)

Fig. 1 Natural endophytic *B. bassiana* isolated from date palm tissues (a) and soil *B. bassiana* isolate that recovered from treated date palm seedlings.

onto quarter-strength PDA plates, which contain 100 μg/mL streptomycin sulphate and 10 μg/mL tetracycline hydrochloride for bacterial inhibition. Plates were incubated at 25 ± 2 °C and 50% RH for 5 -7 d allowing for spore forming. Spore suspensions were made by adding 3-4 drops of sterile distilled water on the fungal colony that were grown on the plate using flame-sterilized loop. The spore suspensions were streaked onto 2% water agar media by using a flame-sterilized metal loop, and plates were incubated under laboratory conditions for 24 h. A single germinated spore was transferred onto full-strength PDA media plate and incubated at ambient temperature [22]. Fungal isolates were stored in 20% glycerol in 10 mL tubes at three temperatures (25, 4, -20 °C) after giving them special cod.

2.4 Molecular Identification of the Isolates

2.4.1 DNA Extraction

DNA was extracted from fungal isolates according to protocol of Graham et al. [25] with minor modification. The weft mycelia was grinded to fine powder using liquid nitrogen and 0.2-0.5 mg was added to 1.5-2 mL eppendorf tube; then, 500 μL of CTAB buffer (2 g CTAB, 1 M Tris pH 8.0, 0.5 M EDTA pH 8.0, 5 M NaCl, 1 g β-mercaptoethanol and 1 g PVP40 pH 5.0) was added and the mix was incubated for 15 min at 55 °C in a recirculating water bath. The mixture was spun at 12,000 ×g (13,000 rpm in microfuge) for 5 min to spin down cell debris, and the supernatant is transferred to clean mircofuge tubes. To each tube, approximately 250 μL of chloroform: iso amyl alcohol (24:1) was added and mixed by inversion. The tubes were spun at 12,000 ×g for 1 min, and the upper aqueous phase transferred to clean microfuge tubes. To each tube 1/10 volume of 7.5 M ammonium acetate was added followed by 1 volume of ice cold absolute ethanol, and washed with 70% ethanol. The DNA was spun into a pellet by centrifugation at 12,000 ×g for 1 min. The DNA then was dried and resuspended in TE buffer pH 8.0 contained RNaseA (10 μg/mL). DNA concentration and quality were determined using NanoSpec-cube (Germany).

2.4.2 PCR Amplification

For identifying *Beauveria* isolates, species-specific PCR primers BbasCG1024F (5'-TGCGGCTGAGGAGGACT-3') and BbasCG1024R (5'-TGCGGCTGAGTGTAGAAC-3') were used according to Ref. [2]. PCR amplifications were performed in a total volume of 20 μL using premix (Bioneer Com., Korea). The mix was prepared via adding 4 μL DNA, 9 μL injection water, 1 μL from each forward and reversed primer, and the already exciting master mix in the tubes 5 μL, completing the reaction to 20 μL. Amplicons were separated by gel electrophoresis in 1.2% agarose gels in TBE buffer (Tris base (890 mM), boric acid (890 mM) and 0.5 M EDTA, pH 8.0), stained with 4 μL ethidium bromide (10 mg/mL) and visualized under UV light. Agarose gel electrophoresis 1.2% was prepared by adding 1.2 g/100 mL TBE buffer. The samples were loaded by adding 5 μL of gel loading buffer and 5 μL of each sample. The gel was run with settings 70 V, 250 A, approximately 90 min, and read after transferring it into the trans-illuminator to be visualized and taking photo. PCR amplification condition for the primer was preformed according to Ref. [2] with denaturation 95 °C for 2 min, 35 cycles at 95 °C (denaturation) for 1 min, 62 °C for 1 min (annealing), 72 °C for 1 min (extension) and a final extension at 72 °C for 5 min using PCR thermocycler (Analytic jena, Germany).

2.5 Screening for Endophytic Fungi

Spore suspensions of MARD 24, 22, 83, 3, 54, 32, 56, 90, 91, 92 isolated from soil and MARD 100 and 108 isolated from plant tissue, were prepared by adding 5 mL of sterile distilled water to pure full growth *B. bassiana* isolate Petri dish and by using a sterile metal scraper. Fungal mycelia were scraped and the solution was poured into a 50 mL Falcon tube

after filtering through sterile miracloth. The spore concentration of *B. bassiana* was determined using haemocytometer and adjusted to 1×10^5, 1×10^7 and 1×10^9 conidia/mL. Date palm seedlings were treated either by spraying directly or stems were injected with the spore suspension using disposable needles. Three replicates were done for each concentration and treatments. Treated seedlings were covered with plastic bags for 24 h and incubated in the rearing room of 25 °C and 70% RH. Three (2 mm) pieces of leaf tissue from each replicate were used to isolate possible endophytic fungi. Pieces were surface sterilized in bleach (1% available chlorine) for 5 min, washed twice in sterile water for 5 min, and then dried by placing them on sterile paper towel. Subsequently, leaf tissue pieces were transferred onto quarter-strength potato dextrose agar (PDA) and incubated as mentioned above before screening for any possible fungal growth.

2.6 Molecular Screening for Endophytic Fungi

Date palm leaf tissues were screened twice, first to investigate the presence of natural enophyta before the injection of isolated endophyta and second to investigate after inoculation if there is any role of these isolates in controlling targeted insect pest. DNA was extracted from plant tissues according to protocol of Graham et al. [25] with minor modification as mentioned previously. Plant tissue samples were collected from all replicates that were treated with the spore suspensions as well, and 0.5 g was grinded to fine powder using liquid nitrogen and added to 2 mL eppendorf tubes before completing DNA extraction process. For investigating the presence of endophytic *B. bassiana* inside plant tissues, species-specific PCR primers: BbasCG1024F (5'-TGCGGCTGAGGAGGACT-3') and BbasCG1024R (5'-TGCGGCTGAGTGTAGAAC-3') were used according to Ferri et al. [26] as mentioned previously. Amplicons were separated by gel electrophoresis in 1.2% agarose gels in TBE buffer (Tris base 890 mM, boric acid 890 mM and 0.5 M EDTA, pH 8.0), stained with 4 μL ethidium bromide (10 mg/mL) and visualized under UV light.

2.7 Laboratory Bioassay Test

The identified endophytic *B. bassiana* isolates (MARD 92, MARD 100 and MARD 108) were tested against *G. mellonella* larvae to measure their pathogenicity. Spore suspension was prepared by adding 5 mL of sterile distilled water to pure full growth fungal isolate Petri dish, and by using a sterile metal scraper, fungal mycelia were scraped and the solution was poured into a 50 mL Falcon tube after filtering the solution through sterile miracloth. Spore concentration was determined using haemocytometer and adjusted to 1×10^5, 1×10^7 and 1×10^9 conidia/mL. Ten larvae (4th-5th) instar in each of the three replicates were sprayed with the suspension and released on 100 g G. *mellonella* rearing medium and placed in 20 cm × 30 cm jars and incubated in the rearing room under 27 ± 2 °C and 75% ± 5% RH [27]. Three replicates for each concentration were used, including control that sprayed with sterile distilled water. Dead larvae were counted every two days.

2.8 Field Efficacy

Fifteen (five for each isolate) mature (about seven years old) date palm trees infested by Dubas bug *O. lybicus* were used for each identified *B. bassiana* enophyta (MARD 92, MARD 100 and MARD 108) and other five trees for the control. 50-75 mL of spore concentration 1×10^9 conidia/mL was injected inside tunnel made 50 cm far from the top (crown) of the tree in the trunk, by using drill, while the control was injected with the same volume of sterile distilled water only. Dubas bug live nymphs/leaflet (10 leaflet/tree) were counted before and after fungal treatment every three days. Percentages mortalities were calculated up to 15 d after treatments. Furthermore, leaves tissues (21 d after injection) were subjected to fungal re-isolation process, followed by DNA extraction and

PCR amplification as mentioned previously.

2.9 Experimental Design and Activity Measures

The laboratory trials were conducted in Biological Control Department of Integrated Pest Management (IPM) Center with complete randomized design (CRD). Field trials were conducted in date palm orchard with randomized complete block design (RCBD), and bio-agents activity was measured according to Schneider & Orel, as Eq. (1):

$$\text{Insecticide activity (\%)} = \frac{\text{mortality in treatment} - \text{mortality in control}}{100\% - \text{mortality in control}} \times 100 \quad (1)$$

3. Results

3.1 Isolation of Natural Endophyte

The result of isolation of natural endophytic isolates of *B. bassiana* from date palm leaves tissues indicated the presence of two isolates marked as MARD 100 and MARD 108. Among soil isolates of *B. bassiana*, only one isolate (MARD 92) showed endophytic activity in both treatments (direct spraying and injection) in the lab screening test. This was proved via re-isolate the fungi from treated seedlings and through applying PCR identification process using the same primers BbasCG1024F and BbasCG1024R.

The identity of entomopathogenic fungal isolated as a natural endophyta and that originally isolated from soil were confirmed by PCR using species-specific primers. One primer was used to determine the presence of the species among a number of samples along with positive and negative control. The presence of a band represents a positive identification as shown by representative gels in Fig. 2. Both two natural isolates and eight out of 10 examined soil isolates were *B. bassiana*. The two isolates that did not express any response to the PCR amplification test using the primers BbasCG1024F and BbasCG1024R were MARD 24 and MARD 83. It can be seen that presence of amplicons bands were at the 838 bp with definite identification according to Ferri et al. [26]. 100 bp DNA marker (Bioneer Com, Korea) was used to mark targeted bands.

3.2 Results of Laboratory Bioassay

The three identified endophytic *B. bassiana* isolates were subjected to quick lab bioassay on *G. mellonella*

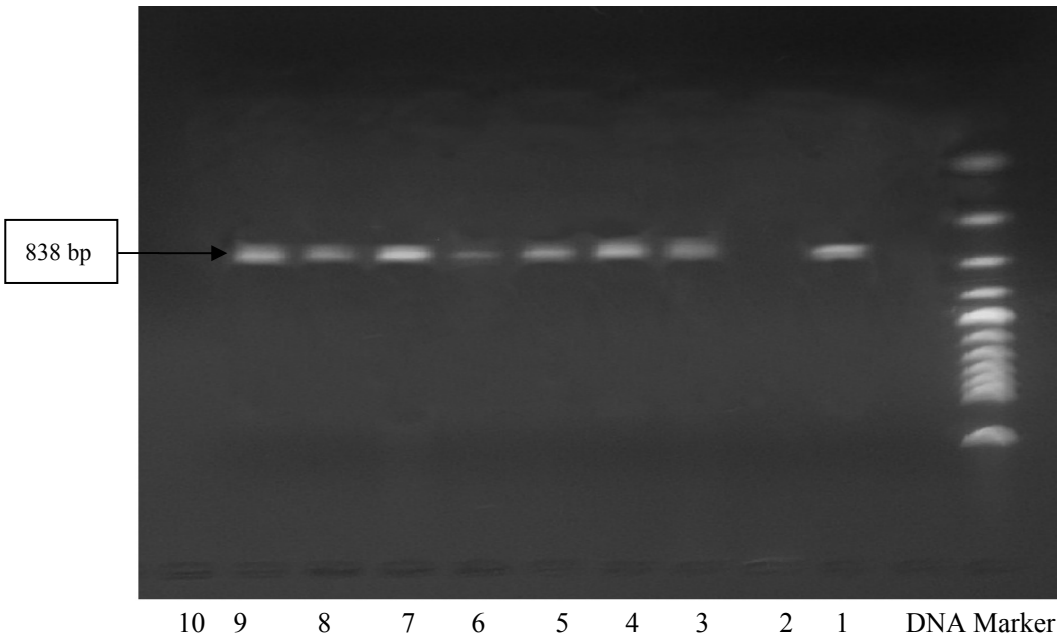


Fig. 2 An 838 bp amplicon using primers BbasCG1024F and BbasCG1024R, representing a positive diagnosis for *B. bassiana* isolated from soil.

Table 1 Mortality rates of *G. mellonella* larvae treated with different concentrations of endophytic *B. bassiana* isolates.

Days after treatment	Mortality rate (%) at different concentration (conidia/mL)		
	1×10^5	1×10^7	1×10^9
MARD 92 isolate (originally from soil)			
2	4.3	5.0	12.2
5	22.0	35.0	30.0
8	35.0	45.0	55.3
11	44.0	55.5	65.5
14	52.6	62.7	85.6
MARD 108 isolate (originally from date palm tissue)			
2	4.0	8.0	12.0
5	20.0	33.0	28.8
8	28.0	44.4	52.0
11	32.0	50.2	68.0
14	51.8	58.0	78.6
MARD 100 isolate (originally from date palm tissue)			
2	3.5	8.6	10.0
5	12.6	22.0	33.3
8	18.0	33.3	52.3
11	32.0	50.5	66.6
14	45.6	66.6	70.8

larvae. The bioassay results (Table 1) revealed that all used isolates have promising biological activity against *G. mellonella*. The highest mortality rates were recorded at the concentration 1×10^9 conidia/mL for all isolates (MARD 92, MARD 108 and MARD 100), reaching 85.6%, 78.6% and 70.8%, respectively, after 14 d from the treatment. However, the lowest mortality rates were recorded at the concentration 1×10^5 conidia/mL, reaching 52.6%, 51.8% and 45.6%, respectively. Soil isolate MARD 92 revealed almost the highest mortality rates after 14 d of treatment at all concentrations (1×10^5, 1×10^7 and 1×10^9 conidia/mL) with 52.6%, 62.7% and 85.6%, respectively, followed by MARD 108 isolate that recorded mortality rate of 51.8%, 58% and 78.6%, respectively, after 14 d from the treatment. The number of dead larvae was increased gradually with time, reaching the highest at the end of the experiment for all examined isolate.

3.3 Results of Field Experiments

According to the lab bioassay results, the concentration 1×10^9 conidia/mL was the best among all endophytic isolates, recording the highest mortality rate (Table 1). Therefore, it was used in the field experiment targeting Dubas bug *O. lybicus* nymphs.

Field experiments results are illustrated in Table 2. It revealed that all endophytic isolates MARD 92, MARD 108 and MARD 100 were efficacious in reducing the number of Dubas bug nymphs in comparison with the control treatment. Isolate MARD 108 scored the highest mortality rate (100%) after 15 d from the treatment. The isolates MARD 100 and MARD 92 expressed mortality rate of 96% and 92%, respectively. It is obvious from the results that mortality percentages were increased with time and reached the highest after 15 d from the treatment.

All 15 date palm trees selected for field experiment test were subjected to the molecular identifying process using the primers BbasCG1024F and BbasCG1024R in order to screen them before injection for any possible presence of endophytic *B. bassiana*. To determine the presence of the injected endophytic *B. bassiana* isolates in the selected date palm trees after injection, DNA was isolated from leaves and amplified using PCR with the same previously

Table 2 Field mortality rates among Dubas bug *O. lybicus* nymphs after injection date palm trees with 1×10^9 conidia/mL endophyte *B. bassiana* isolates.

Isolates code	Percentages of mortality (%) and activity (%) after treatment									
	3 d		6 d		9 d		12 d		15 d	
	Mortality	Activity	Mortality	Activity	Mortality	Activity	Mortality	Activity	Mortality	Activity
MARD 92	3.2	3.2	12.0	12.0	33.0	27.1	72.0	64.1	92.0	88.1
MARD 100	7.0	7.0	10.5	10.5	30.0	23.9	80.0	74.4	96.0	94.1
MARD 108	3.0	3.0	8.6	8.6	42.5	37.5	76.5	69.9	100.0	100.0
Control	0.0	-	0.0	-	8.0	-	22.0	-	33.0	-

The activity was measured according to Schneider & Orel equation, as Eq. (1).

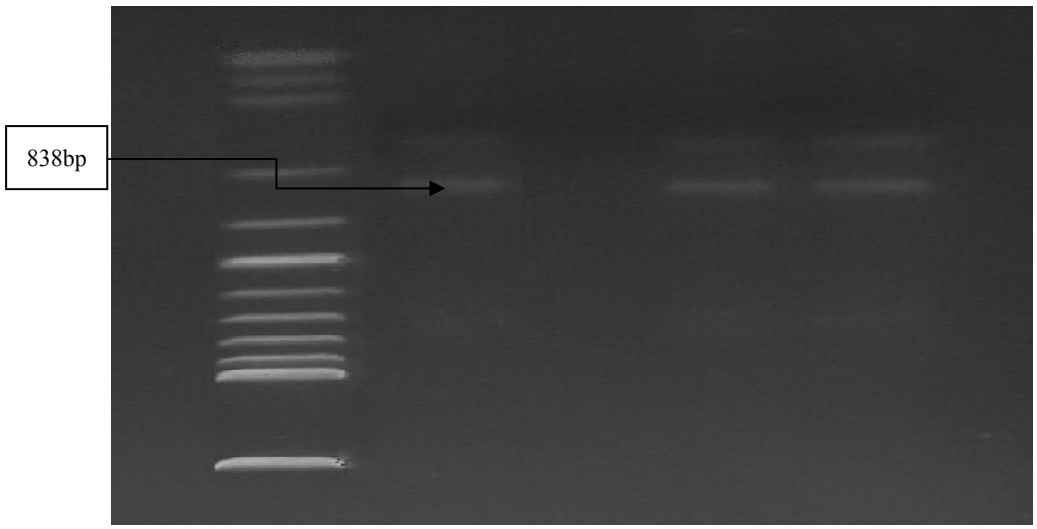


Fig. 3 An 838 bp amplicon using primers BbasCG1024F and BbasCG1024R, representing a positive diagnosis for *B. bassiana* isolated from plant tissues.

mentioned primers after 20 d from the injection date. The presence of the primer band (BbasCG1024F and BbasCG1024R) in the plant tissues DNA injected with MARD 92, MARD 108 and MARD 100 isolates represents a positive identification in comparison with the control, as shown by representative gels in Fig. 3.

4. Discussion

Three isolates of *B. bassiana* were identified having endophytic property. Two of them were naturally isolated from date palm leaves tissues and the third one was from 10 soil isolates. The identity of endophytic *B. bassiana* isolates was confirmed by applying molecular technique using species-specific primer BbasCG1024F and BbasCG1024R that provide reliable identification results according to Ferri et al. [26]. The applied primer was used to amplify isolated DNA directly from fungal growth, which used successfully for the first time to identify *B. bassiana* fungus inside plant tissue.

The result also demonstrated the successful establishment of endophytic *B. bassiana* isolates in date palm leaves after trunk injection. Confirmation was done via applying molecular technique, particularly the primer BbasCG1024F and BbasCG1024R after isolating fungal DNA from the leaves of the injected trees and comparing it with the screening results before inoculation and with the controls. Many previous studies confirmed the establishment of *B. bassiana* as an endophyte in many different plants, for example, in *Theobroma gileri* in Refs. [4, 7]. Bills and Polishook [3] also confirmed

the fungal establishment in seeds and needles of *Pinus monticola*. It was also found in banana tissue cultures by Quesada-Moraga et al. [9]. Maize was another plant where endophytic *B. bassiana* was established via epidermis [4]. Moreover, *B. bassiana* was reported as an endophyte in sorghum leaves, stems and roots [11].

Field experiment results revealed that all the endophytic *B. bassiana* isolates (from plant and soil) expressed high level of mortality rate of targeted Dubas bug nymphs on date palm trees, reaching over 90% at concentration 1×10^9 conidia/mL, which is significantly higher than that in control which was injected with sterilized water only. Many studies found that endophytic *B. bassiana* could protect plants against insect pests. It was reported in Refs. [14, 28, 29] that injecting date palm seedlings with endophytic *B. bassiana* can increase mortality of red palm weevil by over 80%. Quesada-Moraga et al. [9] also mentioned that endophytic *B. bassiana* has a promising possibility of controlling *Timaspis papaveris* (Hymenoptera: Cynipidae). Endophytic *B. bassiana* can inflict high mortality rate among European corn borer (*Ostrinia nubilalis*) [7]. Greenfield et al. [11] mentioned that *B. bassiana* has a promising ability to control sorghum stem borers.

Endophytic *B. bassiana* colonization can be affected by inoculation method, fungal isolate and plant species [11]. Many studies referred to the fungal direct injection as the most successful method, such as in coffee [30], date palm [14], maize [4, 6, 7] and as dipping banana tissue culture in conidial suspension [10] and opium poppy [9].

5. Conclusions

In this study, endophytic *B. bassiana* from date palm tissues and soil was successfully isolated and identified. Endophytic isolates demonstrated high field efficacies in controlling Dubas bug. Therefore, the expansion of the survey for detection of indophyte in date palm trees at different locations to measure their efficacies was recommended.

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