

MOLECULAR IDENTIFICATION AND BIOMASS PRODUCTION OF AN ENDOPHYTIC *Beauveria bassiana* ISOLATED FROM CUCUMBER LEAVES IN IRAQ

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ABSTRACT

Beauveria bassiana as an endophyte fungus was isolated for the first time from cucumber leaves in Duhok province, Kurdistan region, Iraq. The identification was based on molecular and morphological analyses. Morphological description matched well with its molecular analysis using ITS (Internal Transcribed Spacer region). Cultivation of *B. bassiana* isolate on rice and wheat grains as liquid and solid media have shown that the weight, sporulation and germination of the fungus differs significantly between two grains, the maximum weight of the fungus achieved when cultured on rice based medium as liquid (cooked rice) compared to wheat based medium. Inverse results recorded in the case of solid media that the maximum weight of *B. bassiana* achieved on wheat compared to rice medium.

KEY WORD: *Beauveria bassiana*, endophytic fungi, biomass production, cucumber leaves.

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INTRODUCTION

The German scientist Heinrich Anton De Bary (1884) used the term an endophyte to define fungi or bacteria that inhabit the internal tissue of the host plant for all or part of their life cycle (Wilson 1995; Hyde and Soyong 2008). They colonize the internal plant tissues beneath the epidermal cell layers without causing any apparent symptoms in the host, living within the intercellular spaces and penetrate the living cells (Strobel 2003). According to Huang *et al.* (2001); Arnold (2007), It has been estimated that there may be as many as one million different endophytic fungal taxa based on ratio of vascular plants of 1:4-5, and mostly belonging to ascomycetes. Few species have been reported from other groups of fungi (Zheng and Jiang 1995; Norilert *et al.* 2018).

Naturally *Beauveria bassiana* grows in soils throughout the world, causing white muscardine disease to wide range of arthropods, but this fungus also has been recorded as an endophyte in many plants as: elm bark (Doberski and Tribe 1980), maize (Vakili 1990; Arnold and Lewis

2005), in the bark of *Carpinus caroliniana* (Bills and Polishook 1991), potato and cotton (Jones 1994), tomato (Leckie 2002), *Theobroma cacao* (Evans *et al.* 2003), in the seeds and needles of *Pinus monticola* (Ganley and Newcombe 2005), and in *Opium poppies* (Quesada-Moraga *et al.* 2006), banana (Akello *et al.* 2007), coffee (Posada *et al.* 2007), sorghum (Tefera and Vidal 2009), wheat, cotton, tomato, bean, pumpkin (Gurulingappa *et al.* 2010), radiate pine (Brownbridge *et al.* 2012), common bean (Ramos *et al.* 2017) and Sugarcane (Donga *et al.* 2018). There is a growing interest in applying endophytic entomopathogens in management of insect pests (Vega 2018). There are two principal mass production technologies; combined method (Sikura and Primak 1970) or diphasic production system (Kassa *et al.* 2008) by using solid material as sorghum, rice, wheat and millet (Leite *et al.* 2002; Machado *et al.* 2010). The cultivation of microorganisms using any cultured technique needs knowledge of nutritional requirements, which are responsible for the mycelia growth and spore yield. The macro elements as carbon, hydrogen, oxygen,

sulfur, phosphorous and nitrogen are required which are the basic components of carbohydrates, nucleic acids and proteins.

The present study was carried out to identify the endophytic *B. bassiana* isolated from cucumber leaves depending on both morphological characteristics and molecular identification and evaluate the two grains; rice and wheat as liquid and solid media for its biomass production.

MATERIAL AND METHODS

Isolation of endophytic fungi

To isolate endophytic entomopathogenic fungi harboring plants, cucumber plants from different villages related to Amadia district (1122 m above sea level) were collected. The plant samples kept in plastic bags and transferred to the laboratory for isolation of the entomopathogenic fungi. For each plant, tissues including leaves, stems and roots were washed by tap water and then surface-sterilized according to Arnold *et al.* (2007). The tissues were well dried on sterile paper towels and the edges were cut to remove dead tissues ensuing from the disinfection process. Five sections of each plant part (root, stem and leaves) were placed in petri dishes of selective media. Four replicates were used/ plant part. The plates incubated at 25°C for two weeks. Fungi growing from tissues were transferred on standard PDA medium ((Himedia laboratories Pvt. Ltd. India). for identification.

Isolates recovered from single conidia were grown as mycelia in 250-ml conical flasks containing 100 ml of potato dextrose broth. Cultures were then shaken and incubated at 25°C for seven days in darkness. The mycelial growth were filtered from liquid culture under aseptic conditions and then frozen at -20°C.

DNA extraction and amplification

The extraction was done according to commercial animal and fungi DNA preparation kit protocol. Genomic DNA was used as template for PCR amplification of ITS region using universal primers ITS5/ITS4 (White *et al.* 1990). The PCR reactions were performed in a final volume of 50 µl containing 25 µl 2x Taq PCR Master Mix, 2 µl of each reverse and forward primer (20 pml), 2 µl of genomic DNA (30-100 ng/ µl) and 19 µl of RNase-Free water. Amplification was performed in a GeneAmp

PCR System 9700 thermocycler (Applied Biosystems) according to a program as follows: 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 45 s and final extension step of 72°C for 10 min.

Amplified PCR products were visualized by 1% agarose gel electrophoresis. The sequencing was performed at Macrogen Company, Korea. The sequences were checked and aligned using BioEdit sequence alignment editor 7.0.0 (Isis Pharmaceuticals, Inc., Carlsbad, CA, USA). The sequence was submitted to GenBank (Genbank accession MH374537). The similarity of the sequence with homologous sequences deposited in GenBank was calculated using the "BLAST" tool on the National Center for Biotechnology Information (NCBI) website. Alignment of selected sequences was done with clustalW. The phylogenetic tree was constructed using the Neighbor-Joining method by Jukes-Cantor model with MEGA7. Branch support was estimated by bootstrap analysis with 1000 replicates.

Mass production of an endophytic *B. bassiana*

Two grains, viz, wheat and rice used for estimating the biomass of the endophytic *B. bassiana* isolate at 25 °C as liquid and solid medium.

Liquid medium:

For each wheat and rice, 20 g of grain washed well and boiled in distilled water for 1 hr. meshed properly and filtered. The size of filtration completed to one liter with distilled water. The grain mediums (250 ml) packed separately in individual conical flask (500 ml) with three replicates/ medium/ dry period. They plugged with cotton wool and autoclaved at 121°C. After cooling, each flask inoculated with 1 ml of the fungal suspension (1×10^7). For control treatment, the sterilized distilled water was inoculated with fungal suspension and then the flasks were incubated at 25°C for 21 days. All these procedures were done under laminar air flow chamber. After 21 days incubation, the flasks were agitated and filtered through filter paper. The fungal mat dried at laboratory temperature to determine the weight, spore concentration and spore germination in each grain medium after 1, 5 and 10 days of drying.

Solid medium:

Amount of rice and wheat grains was washed and soaked with tap water for 2hr (rice grains) and 12hr (wheat grains), then the excess water

drained completely. One hundred gram of each grain was put in 500 ml flasks. To each of these flasks, 2g of calcium carbonate and 2g of calcium sulphate was added and mixed thoroughly to get uniform coating of salts over grains. This process will help in preventing the grain particles sticking together and thereby providing more surface area for fungal growth. The flasks plugged with cotton wool and autoclaved at 121°C for sterilization. After cooling, each flask was inoculated with 1 ml of the fungal suspension (1×10^7) under aseptic conditions and incubated for 21 days at 25°C for the production of aerial conidia. Three replications / medium/ dry period were used. After 21 days of incubation, grains with fungal growth were dried under aseptic conditions at lab temperature to record the mass of fungus, spore production and spore germination after 1, 5 and 10 days of drying date.

The data were statistically analyzed by SAS program using Complete Randomized Design (CRD) with three replicates and the means were compared using Duncan's multiple range tests at $P \leq 0.05$.

RESULTS

Identification

The results of rDNA-ITS sequencing of the *Beauveria* isolate showed 609 bp of special DNA fragments sequenced. Using blast search, to compare the resulting sequence with sequences of rDNA accessed in Genbank, phylogenetic analysis showed that the obtained sequences shares 99% homology to *Beauveria bassiana* strains: Chinese isolates (KP994951, MG844431, MG844430); Spanish isolate (KC753391); South Africa isolates (MF802492, JX110376); Turkish isolate (FJ177439) and Mexico isolate (JN379811) (Fig.1). Together, morphological and molecular identification showed that our *Beauveria* isolate is *B. bassiana* (Genbank accession No. MH374537).

Mass production

Liquid medium

The results showed that the mycelia weight for *B. bassiana* was significantly affected by the type of media (Tab.2) and also the weight was decreased after each drying period. Between the two liquid media, rice medium produced maximum biomass; the highest weight of mycelia biomass reached to 0.72g/ 250 ml of medium which recorded with rice after one day drying and significantly not differ with the weight

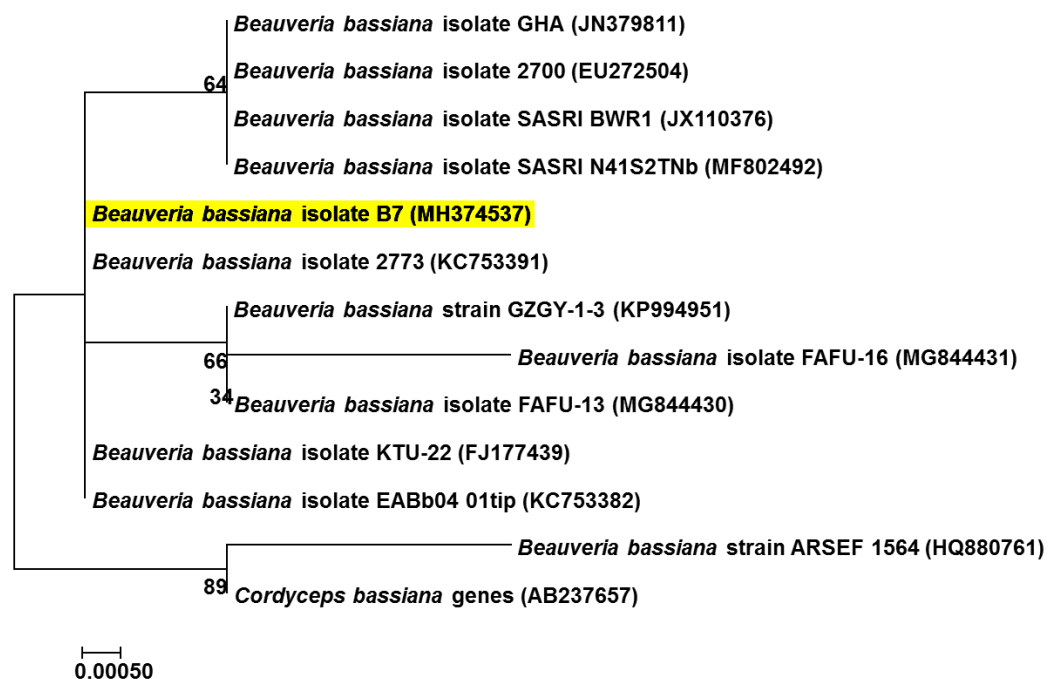


Fig. (1):Phylogenetic tree of *Beauveria bassiana* based on Neighbor-Joining analysis with 1000 bootstrap replicates of ITS-rDNA sequences of the new strain from Iraq (in yellow) and related *Beauveria* species from GenBank. GenBank accession numbers provided behind the species names.

recorded after 5 and 10 days drying on the same medium as 0.68 and 0.67g/ 250 ml medium. The lowest weight was 0.55 g/ 250 ml recorded on wheat liquid medium. The *B. bassiana* was also produced a significantly much higher amount of conidia when cultured with rice medium as 6.1×10^6 conidia/ ml suspension

after ten day drying compared to 4.1×10^5 on wheat medium for the same drying period (Tab 2). The results also showed that the concentration of conidia/ ml was increased gradually after each drying period but significantly not differ.

Table (2): Biomass production of *Beauveria bassiana* on wheat and rice grains as liquid medium

Medium	Drying period (Days)	Weight gm./ 250 ml culture	Concentration Conidia/ml	Germination %
Wheat	1	0.63 ± 0.02 b	1.2×10^5 b	99.67 ± 4.84 a
	5	0.61 ± 0.02 b	2.6×10^5 b	97.33 ± 2.60 a
	10	0.55 ± 0.01 b	4.1×10^5 b	91.33 ± 0.58 ab
Rice	1	0.72 ± 0.02 a	2.2×10^6 a	82.12 ± 2.87 b
	5	0.68 ± 0.05 ab	4.6×10^6 a	80.33 ± 2.67 b
	10	0.67 ± 0.02 ab	6.1×10^6 a	80.09 ± 2.89 b
Control		0.00 c	0.00 c	0.00 c

Means followed by a common letters within the same column are not significantly differed at 5% level by DMRT.

Between the two liquid medium, conidia produced on wheat medium recorded the highest percentage of germination (Tab 2) which reached to 99.67%. With liquid media based on wheat and rice, *B. bassiana* produced conidia,

blastospores (hyphal bodies) and hyphae (Fig. 2). Microsclerotia and newly reproduced conidia from hyphae were also observed within a short time. Among all the fungal cells, conidia with germ tubes treated as germination.

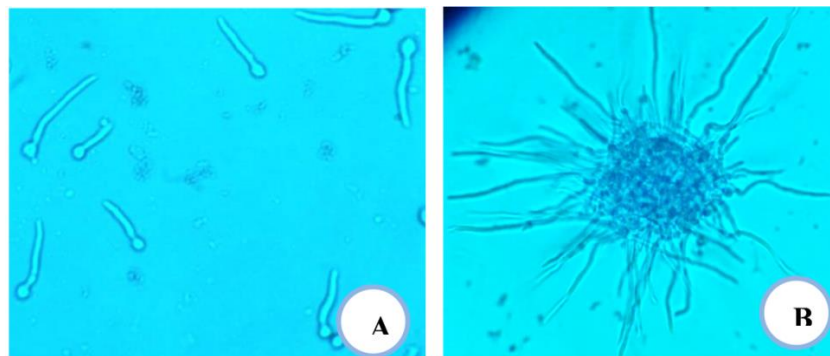


Fig. (2): Various morphologies as outcomes of germination of *B. bassiana* conidia (A: elongation of germ tube, B: Microsclerotia, scale bar A-B= 5µm.

Solid medium

The largest weight of fungal dust was recorded on wheat grains after 10 days drying as 3.00 g/ 100g grains, and significantly differs with the weight recorded on rice grains as 2.38g/ 100g grains. It's clear from data analysis, that the drying period had a significant effect on the

weight of spore dust, the weight was increased by increasing the drying period, and the lowest weight was recorded after one day drying as 1.11 g/ 100g rice grains. This increasing of weight may be due to that the drying helps to separate the spores from the grains (Tab. 3).

Table (3): Biomass production of *Beauveria bassiana* isolates on wheat and rice grains as solid medium

Grains	Drying period (Days)	Weight gm./100gm. culture	Concentration Conidia/ml	Germination %
Wheat	1	1.11 ± 0.13 c	2.7 X 10 ⁶ b	90.33 ± 4.88 a
	5	2.60 ± 0.21 a	3.0 X 10 ⁶ b	89.00 ± 2.08 a
	10	3.00 ± 0.21 a	5.0 X 10 ⁶ a	88.33 ± 3.03 a
Rice	1	1.13 ± 0.11 c	1.4 X 10 ⁵ d	70.33 ± 3.28 b
	5	2.13 ± 0.19 b	3.5 X 10 ⁵ d	57.33 ± 3.53 c
	10	2.38 ± 0.19 ab	8.3 X 10 ⁵ c	46.00 ± 2.31 d
Control		0.38 ± 0.02 d	0.00 e	0.00 e

Means followed by a common letter within the same column are not significantly different at 5% level by DMRT.

The concentration of conidia/ ml of fungal suspension were significantly affected by the type of media and not differ according to the drying periods. The highest spore production was recorded with *B. bassiana* cultured on wheat grains as 5.0 x 10⁶ conidia/ ml after 10 days drying compared to 8.3 x 10⁵ when cultured on rice grains. The lowest rate of germination was 46.00% after 10 days drying recorded with *B. bassiana* cultured on rice grains compared to 88.33% when cultured on wheat grains.

DISCUSSION

Beauveria bassiana along with other entomopathogenic fungi was previously isolated from the Iraqi soil (Assaf, 2007; Assaf *et al.* 2011; Abdullah and Mohamed Amin, 2009; Abdullah *et al.* 2015) which depended on morphological characteristics for species identification. In this study *B. bassiana* as an endophytic isolated from cucumber leaves is newly reported in Iraq and identified by ITS-rDNA based phylogenetic analysis which is a sufficient marker for identifying *B. bassiana*

from closely related species (Tuand Kirschner 2014).

Cultivation of endophytic *B. bassiana* isolate on rice and wheat grains as liquid and solid media have shown that the fungal weight, sporulation and germination differs significantly between two grains. *B. bassiana* achieved the maximum weight and sporulation when cultured on rice based medium as liquid (cooked rice) compared to wheat based medium. Inverse results recorded in case of solid media, that the maximum weight of *B. bassiana* achieved on wheat compared to rice medium. The two different substrates (wheat and rice) supported growth of the of *B. bassiana*, confirming the earlier work (Ibrahim and Low 1993; Sergio et al. 2003; Feng et al. 2004) that fungi can be cost-effectively mass-produced on different solid substrates. Bhaduria et al. (2012) who used 15 various grains as solid media for *B. bassiana* mass production recorded a dry weight of fungus as 0.378 and 0.555g/ 100g of wheat and rice grains, respectively. Ibrahim et al. (2015) stated that the *B. bassiana* dry weight per 100gram of substrate was highly variable and recorded a weight as 20.7, 5.0 and 1.3g/ 100g for burgul, rice, and wheat, respectively. Latifian et al. (2013) reported that *B. bassiana* cultured on liquid medium of rice produced significantly higher spore production (3.3×10^7 conidia/ ml) compared to conidia produced on liquid wheat medium (5.1×10^6). Goulet et al. (2014) reported that an increase in the depth of the liquid biomass in the cultivation container led to decrease in the yield of aerial conidia/ ml of initial fungal biomass.

Conidia produced on wheat medium recorded the highest percentage of germination which reached to 99.67% after one day of drying. The rate of germination is an important factor in the infection process, with faster germinating conidia generally considered to have a greater chance of causing infection. However, germination rates on the cuticle may not reflect those seen on artificial media because of the presence of anti-fungal compounds associated with the cuticle (Charnley 1989). Bin and Mitsuaki (2006) revealed that *B. bassiana* could germinate and extend hypha in sterilized distilled water. They recorded, just one-week after inoculation, visible long mycelia but the new mycelia showed degradation after 4 weeks survival; perhaps because of exhaustion

of nutrients stored in the conidia. They also mentioned that the nutritional conditions in water are thought to be poorer compared with soil, that may limit the germination of *B. bassiana* in sterilized water, and keep it at a low level. The conidia may quickly lose their viability when they exhausted their own stored nutrients.

With liquid media based on rice and wheat, *B. bassiana* conidia produced germ tubes, blastospores (hyphal bodies) and hyphae. Fargues et al. (1979) reported that both *B. bassiana* and *B. brongniartii* had the ability to produce blastospores in liquid media, but it is difficult to storage and kept their viability for long time (Ferron 1981). Brown et al. (1988) reported that the fungal biomass is increased via vegetative growth forming either hyphal filaments, often with copious branching, or various forms of pellets of mycelia depending on the strain, medium, and culture parameters. The majorities of fungal isolates are also capable of forming single cells via schizolytic separation at the septa or mechanical fragmentation of the hyphae, and can also be generated from the hyphae by yeast-like budding (Jackson et al. 1997). In general, nutrient-rich media containing high concentrations of carbon and nitrogen sources are more likely to produce larger quantities of these vegetative propagules (blastospores, hyphal bodies, mycelium, and microsclerotia). Blastospores of *B. bassiana* and *I. fumosorosea* have been obtained in liquid cultures amended with high concentrations of glucose, under high aeration rates, and an appropriate source of nitrogen to yield high concentrations of blastospores (Jaronski and Mascarini 2013; Mascarini et al. 2015).

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پشکینا گهردیله یی و و زیده کرنا گهروویئ نخافتی *Beauveria bassiana*(Bals.) Vuill. ژ به لکین خیاری ل عیراقئ

پوخته

گهروویئ *Beauveria bassiana* هاته تومارکرن وهک گهروویئ نخافتی بو جارا ئیکئ ژ به لگین خیاری ل پاریزگه ها دهوکیل عیراقئ. پشکینا مورفولوجی و گهردیله یی بکارئینانا ITS-rDNA بو گهروویئ هاته کرن. د ئه نجامئین زیده کرنا گهروویئ *Beauveria bassiana* لسهر دنکئین برنجی و گه نمی وهک خارنئین شل و هیشک جوداهیین مه عنه وی د کیشه یا مایسیلیومیو ریژا چیکرن و شینبونا سپورا دا هه بون کو زیده ترین کیشه یا مایسیلیومی هاته تومارکرن لسهر برنجی وهک خارنا شل به رامبه ری گه نمی. به لئ وهک خارنئین هیشک زیده ترین کیشه یا مایسیلیومی هاته تومارکرن لسهر گه نمی به رامبه ری برنجی.

التصنيف الجزيئي و اکتار الفطر الکامن *Beauveria bassiana*(Bals.) Vuill.

من اوراق نبات الخیار في العراق

الخلاصة

تم تسجيل الفطر *Beauveria bassiana*(Bals.) Vuill. كفطر کامن لاول مرة من اوراق نبات الخیار في محافظة دهوك/ اقليم كوردستان/ العراق. تم التصنيف اعتمادا على الصفات المورفولوجية و التحليل الجزيئي باستخدام ITS-rDNA. عند اکتار الفطر على حبوب الرز و الحنطة كأوساط سائلة و صلبة سجلت اختلافات معنوية في وزن المایسیلیوم، انتاج و انبات الكونیدات حيث سجل أعلى وزن للفطر على الوسط السائل للرز مقارنة بالوسط السائل للحنطة. في حالة الاوساط الصلبة فأنتائج كانت عكسية حيث سجل أعلى وزن على الحنطة مقارنة بالرز.