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ISOLATION, IDENTIFICATION AND PATHOGENICITY ASSESSMENT OF A NEW ISOLATE OF ENTOMOPATHOGENIC FUNGUS, *BEAUVERIA BASSIANA* IN IRAN

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Abstract: A new isolate (BEH) of entomopathogenic fungus, *Beauveria bassiana* was isolated from soil using DOC2 selective medium. This isolate was characterized by conidiophores consisting of whorls and dense clusters of short conidiophorous cells with one-celled spherical conidia. Conidial length and width were 2.27±0.22 µm and 1.85±0.32 µm, respectively with length/width ratio of 1.23. Colonies on SDYA medium were normally white to pale yellow and sometimes red pigmented in reverse. Because of importance of this pathogen in biocontrol programs around the world and difficulties with morphological identification, a molecular technique was developed to assist complementary identification of the fungus. Pr1, a pathogenicity-related alkaline cuticle-degrading serine protease, with defined sequence in *B. bassiana* was amplified using PCR technique. The presence of this gene in isolated fungus (BEH) with 744 bp sequence length, as visualized on agarose gel affirmed the data from morphological studies that the new isolate (BEH) pertained to entomopathogenic fungus, *B. bassiana*. Pathogenicity of new isolate against *Tenebrio molitor* and its recovering was the other confirmation that the isolated fungus belonged to *B. bassiana*, using further light microscope studies.

Key words: *Beauveria bassiana*, Pr1, PCR, identification, morphologic, virulence

INTRODUCTION

Current problems concerning the use of synthetic chemical insecticides gave rise to a sense of urgency for the development of biological control agents as supplements or alternatives to these chemicals (St. Leger *et al*. 1996). Entomopathogenic fungi are key regulatory factors in pest insect populations in nature (Charnley 1997), are attracting attention as potential biological control agents (Clarkson and Charnley 1996), and provide the only practical microbial control of insects that feed by sucking plant or animal juices, and for the many acridid (grasshoppers and locusts) and coleopteran pests which have no known viral or bacterial diseases (Hajek and St. Leger 1994; St. Leger *et al*. 1996). Some of the hyphomycetous entomopathogenic fungi developed as promising alternatives for chemical insecticides (Wang *et al*. 2003). The fungus *Beauveria bassiana* (Bals.) Vuill. has a wide host range (Clarkson and Charnley 1996), is widely distributed in all regions of the world and can be isolated from insects, mites, and soil, where it is a part of the normal microbial flora, and other substrates (Boucias and Pendland 1998). This species has a large genetic variation among different isolates. Pathogenicity and virulence to different arthropods as well as enzymatic and DNA characteristics vary among different isolates (Almeida *et al*. 1997; Moino *et al*. 1998). Several isolates of *B. bassiana* were formulated and registrated as commercial products against a wide range of insect pests (Feng *et al*. 1994).

Exact identification of fungal species has major importance before doing any further experiment and is very difficult and sometimes impossible by means of morphological characteristics. Morphological criteria are generally used to identify and classify *Beauveria* spp. Distinction between *Beauveria* species has always been problematic, possibly due to the comparatively large heterogenicity of spherical *B. bassiana* (Glare and Inwood 1998). Traditionally, the main difference between the most common species, *B. bassiana* and *B. brogniartii*, is the shape and size of conidia, with the former having mainly spherical conidia and the latter more cylindrical conidia (Brady 1979a, b). Some researches showed that spore shape could alter after culturing (Townsend *et al*. 1995). Because of these difficulties with identification, a number of molecular techniques were developed to assist isolate and/or species identification in *Beauveria* (Kosir *et al*. 1991; St. Leger *et al*. 1992; Hegedes and Khachatourians 1993, 1996; Bidochka *et al*. 1994; Maurer *et al*. 1997).

Entomopathogenic fungi including *B. bassiana* produce proteases, chitinases, and lipases which can degrade insect cuticle (Charnley and St. Leger 1991; Charnley 2003). Ingress through the cuticle is facilitated by a combination of mechanical force and enzymic degradation (Zacharuk 1970; Hassan and Charnley 1989). An extracellular alka-

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line serine protease from *B. bassiana* (Prl) was characterized with high activity against the insect cuticle (Bidochka and Khachatourians 1987; St. Leger *et al*. 1987) and its genetic structure and nucleotide sequence was reported (Joshi *et al*. 1995; Kim *et al*. 1999). PCR amplification of Pr1 gene was considered as another way for detection. Finally, pathogenicity and virulence of fungal isolate were assayed to find out the ability of fungus to establish the disease and death in insect host (entomopathogenicity). To give a description of new isolated isolate of the entomopathogenic fungus, *B. bassiana*, to eliminate the confusion over species assignment of newly isolated fungus, and to introduce a reliable method for species this purpose, we initiated a study utilizing morphological measurements and simple molecular technique and virulence determination to identify a new fungus for establishing later experiments.

MATERIALS AND METHODS

Isolation of fungus from soil

Selective medium is generally required for isolation of *B. bassiana* from soil. DOC2 medium that contained no dextrose and consisted of 3 g Bactopeptone, 0.2 g $CuCl_2$, 2 mg crystal violet, 15 g agar and 1 000 ml distilled water (Shimazu and Sato 1996), was used for isolation of the fungus from soil specimens, pH was adjusted to 10 using $Na_2 CO_3$ and HCl, autoclaved at 120°C for 20 minutes and poured into 9 cm Petri dishes. Soil sample (1 g) from a corn field in Behshahr, Mazandaran, Iran was suspended in sterile distilled water (200 ml) containing 0.03 per cent Tween® 80 as surfactant. Suspensions were applied at a concentration of 0.2 ml/plate and spread using a glass rod. Plates were incubated at 25°C in the total darkness. Colonies obtained from each plate were transferred to Sabouraud's dextrose agar plates supplemented with 1% yeast extract (SDYA) for primary morphological identification. For inhibition of any variation in colony, spores were spread on SDYA plates and a single spore was transferred to new SDYA plate to constitute the main source of fungal inoculum in further experiments.

Extraction of DNA

A known isolate, DEBI008, was included as standard in the experiments. This isolate was isolated from a locust, *Chortippus brunneus* (Orthoptera: Acrididae), from Iran. SDB (Sabouraud's Dextrose Broth) medium consisting of 2% glucose, 0.5% peptone, and 0.5% yeast extract was used as a liquid medium for fungal growth. 100 ml flasks containing 50 ml of sterile medium were inoculated with conidia from 14-day old SDYA cultures and incubated at 120 rpm in an orbital shaker at 27°C. After 4 days, mycelia were harvested using No. 1 Whatman filter paper and Buchnel funnel, wrapped in tin pockets and ground to fine powder in liquid nitrogen using mortar and pestle. Ground mycelia were transferred to 1.5 ml microtubes and DNA extraction was carried out with some modifications from Möller *et al*. (1992). 450 µl of TES [1.4 M NaCl; 0.1 M Tris-HCl, pH 8.0 and 20 mM EDTA (ethylendiamintetraacetic acid)], 50 µl of 20% CTAB (cethyltrimethylammonium-bromide), and 2 µl of ß-mercaptoethanol were added to each tube and thoroughly mixed. Tubes were placed in water bath in a polystyrene holder at 65°C for 90 minutes then tubes were centrifuged (in Sanyo, Harrier 18/80) in centrifuge at 5 000 rpm for 5 minutes after adding chloroform : isoamyl alcohol. Volume of the supernatant was doubled by adding 10% of ammonium acetate and 90% isopropanol. Then they were placed on ice for 30 minutes and centrifuged at 12 000 rpm for 10 minutes. Supernatants were aspirated off leaving the DNA pellet in the tube. The pellet was washed twice with cold 70% ethanol and desiccated at 37°C for 10 minutes. Then it was dissolved in 50 µl TE buffer (10 mM Tris-HCl and 1 mM EDTA), agitated gently, and left in a fridge (4°C) overnight. EDTA, CTAB and ß-mercaptoethanol were from Sigma (UK) and Tris-base was from Promega (USA). Extracted DNA was qualified on agarose gel [1.5% in 0.5 m TBE buffer (Tris-HCl, boric acid, and EDTA)] stained with 5 µl ethidium bromide (Sigma). 5 µl of extracted DNA was mixed with a tiny drop of loading buffer and poured in each well of agarose gel and subject to the constant voltage of 30 V for 45 minutes (Bio-Rad, POWER PAC 3 000).

Primers design and PCR conditions

Gene structure and nucleotide sequence of Pr1, an insect cuticle degrading serine protease from *B. bassiana* was published (Joshi *et al*. 1995; Kim *et al*. 1999). Polymerase Chain Reaction (PCR), a molecular biological technique for creating multiple copies (amplifying) of DNA without using a living organism, was used for amplification of Pr1 gene to confirm the presence of this gene in isolated fungus. Primers, artificial DNA nucleotides that exactly match the beginning and end of DNA fragment to be amplified, were designed according to the cDNA sequences of *B. bassiana* Pr1 (Kim *et al*. 1999) using the program Primer3 and obtained from (Sigma Genosys, UK). These primers (forward, 5′-GCACCCTCAACCAAGAACA-3′ and reverse, 5′-TAGTCCCACCACCAATCCA-3′) covered a region including 744 bp as fragment DNA for amplification.

PCR reaction mixture (25 µl) was consisted of 25 mM 2x buffer, $MgCl_2$, 0.25 µl from each of dNTPs, 0.2 µl of each primer, 5 U/µl Taq Polymerase (all from AB gene, UK), 2 µl of genomic DNA and double distilled water. The PTC-200 thermal cycler (MJ Research) was programmed as follows: 4 min at 95°C for initial denaturation; 37 cycles of denaturation for 40 s at 94°C, annealing for 40 s at 58°C, extension for 45 s at 72°C; and a final extension for 6 min at 72°C.

Virulence of fungal inoculum

As the fungus *B. bassiana* is an entomopathogen, the ability of fungal isolates to cause infection and disease in insects and recovering the fungus from dead insect bodies were used as another parameter in completing the identification process. DEBI008 and new isolate were used in this bioassay. Fully sporulated 14-days-old SDYA Petri dishes were selected for fungal inoculum preparation. Conidia were scrapped from dishes in the sterile condition in microflow and transferred to a sterile 100 ml flask. Ten ml of sterile distilled water containing 0.03 per cent Tween® 80 was added to each flask as surfactant and

agitated gently for 15 min. Concentration of conidia in each suspension was estimated using Improved Neubauer Haemocytometer (Weber Scientific International Ltd., UK) and adjusted to 10^7 conidia/ml. Thirty fourth instar larvae of mealworm, *Tenebrio molitor* (Coleoptera: Tenebrionidae) were dipped in each suspension for 30 s. Control larvae treated with distilled water containing 0.03 per cent Tween® 80. Batches of ten larvae placed in each 9 cm diameter plastic Petri dishes covered with wet (1 ml) No. 1 Whatman paper. Experiment was repeated twice. Dishes were incubated at 26±1°C in an incubator in darkness and mortality of insects was recorded for ten days. Dead larvae were removed daily and placed in new Petri dishes covered with wet Whatman paper for fungal emergence. Conidia from dead insects of each isolate were transferred separately to SDYA medium in a sterile condition. Where needed selective medium (Oatmeal Agar medium) was used for isolation of fungus. Light microscopic studies and colony form proved that recovered fungus is the same as inoculated fungus, *B. bassiana*.

RESULTS

There were one to five small colonies on DOC2 that resembled to *B. bassiana*. Transferred conidia on SDYA and colony form on plates primarily showed that isolated fungus may belong to *Beauveria* genus and in particular to *B. bassiana* species. Single spore colony was made to prevent any variation in colony. Isolate was characterized microscopically (ZEISS, 40x) by conidiophores consisting of whorls and dense cluster of sympodial, short and globose conidiogenous cells with apical zig-zag appearance and one-celled spherical conidia. Conidial length and width were 2.27±0.22 μm and 1.85±0.32 μm, respectively with length/width ratio of 1.23. Colonies on SDYA were normally white to pale yellow and sometimes red pigmented in reverse.

When the DNA was detected in extracted materials (profile has not shown), a part of *pr1* gene was used for amplification with defined primers. The result of PCR on the gene of consideration has been shown in figure 1. DEBI008 (as control isolate) and new isolate (BEH) produced the same size PCR product. As can be seen from figure 1, the gene product bands have been visualized at around 750 bps on agarose gel.

Results of fungal virulence towards host insect for DEBI008 (as control isolate) and new isolate (BEH) have been presented in figure 2. New isolate caused disease and death in insects as DEBI008 did. So, new isolate had the ability to pass through the host cuticle and use haemolymph nutrients. Mean lethal time (LT_{50}) is an accepted determinant for the virulence of chemical insecticides and biocontrol agents of insects. As can be in figure 3, LT_{50} value for DEBI008 and new isolate was calculated 2.72±0.3 and 4.38±0.6 days, respectively. These data showed that BEH (new isolate) was less virulent than DEBI008 towards *T. molitor*. After host death and utilization of all its internal nutrients, fungus emerged from insect body and produced aerial mycelia and conidia on it (Fig. 3). This emergence suggested that the fungus has caused the death and is an entomopathogen.

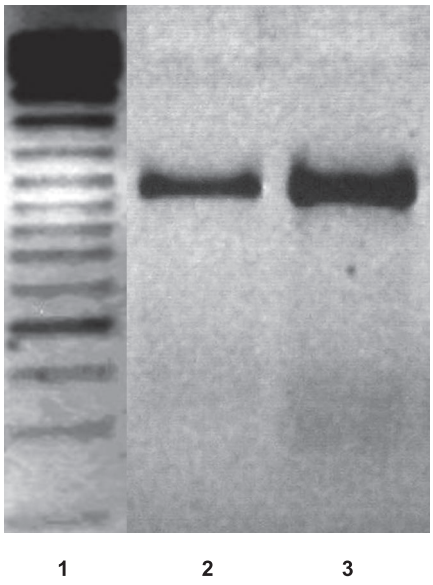

Fig. 1. Agarose gel profile of PCR amplification of pr1 gene in two isolates of *B. bassiana*. 1: Marker (standard), 2: DEBI-008, 3: BEH

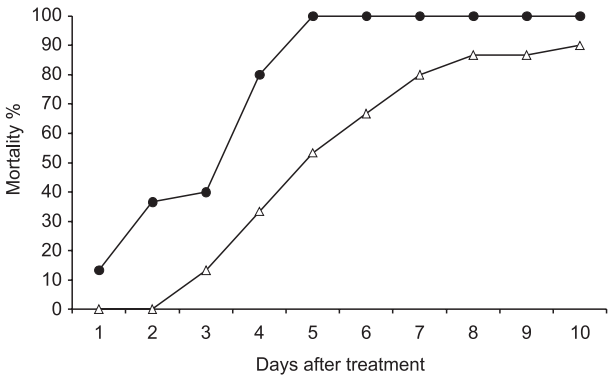

Fig. 2. Mortality (%) of *T. molitor* larvae treated with conidial suspension (10^7 conidia/ml) of new isolate (BEH) and DEBI-008 within ten days after treatment

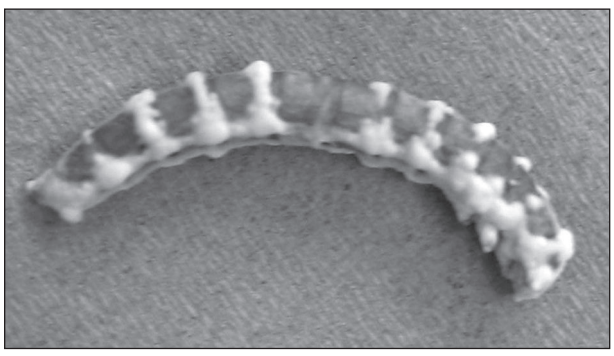

Fig. 3. Emergence of *B. bassiana* (isolate BEH) from dead larvae of *T. molitor*

DISCUSSION

Ability of *B. bassiana* to grow in adverse conditions with poor nutrition and varoius pH makes DOC2 medium a potential medium for fungus isolation. This fungus can germinate and grow in a wide range of pH from four to 11 (Shimazu and Sato 1996). However, most fungi prefer a more or less acidic media. High pH along with addition of $CuCl_2$ caused the inhibition of saprophytic fungus *Penicillium* spp. but did not prevent the *B. bassiana* germination and development on DOC2. This emphasized the results of Shimazu and Sato (1996) for high capacity of *B. bassiana* for growing in various conditions. The used selective medium provided severe conditions for *B. bassiana* which were even more severe and inhibitive for other fungi.

Morphological characteristics described here were in agreement with those have been defined by Samson *et al*. (1988) and this isolate appeared typical of those described elsewhere. Although morphologic criteria are the necessary characteristics for interspecific and intraspecific discrimination, it should be confirmed with other characteristics.

Total genome size of *B. bassiana* has been determined to be 34.3-44.1 mb (Viaud *et al*. 1996). This large weight of DNA can be the cause of its precipitation just near the wells of agarose gel. PCR amplification is a very sensitive process and needs to be optimized for desirable results. Concentration of DNA and particularly annealing temperature of primers has high importance in successful PCR amplification. For this optimization, temperature gradient of annealing temperature from 56°C to 65°C was used in thermal cycler and 58°C was showed to be the optimum temperature. Wang *et al*. (2003) similarly used this temperature as optimum temperature for amplifying the *pr1* gene. But, Viaud *et al*. (1996) used the 65°C as annealing temperature. In addition, high concentrations of DNA can be problematic with practically no desired product. PCR amplification showed the similarity of an extensive part of *pr1* gene structure in DEBI008 with new isolate and in both of them with those has been presented by Joshi *et al*. (1995) and Kim *et al*. (1999). Existence of *pr1* gene in newly isolated fungus emphasized that this fungus is an isolate of entomopathogenic fungus *B. bassiana*.

Because of importance of *B. bassiana* in biocontrol programmes around the world and difficulties with morphological identification, a number of techniques have been used to assist identification of *Beauveria* (Glare and Inwood 1998). Mugnai *et al*. (1989) using morphological and biochemical tests for separating *Beauveria* species found that *B. bassiana* was relatively heterogenous in most tests and produced both spherical and ellipsoidal conidia. They concluded that spore size was variable *in vitro* and should be used in conjunction with other characteristics. Furthermore, ellipsoidal conidia can be produced on the host, but only spherical conidia in culture (Townsend *et al*. 1995; Glare and Inwood 1998). Glare and Inwood (1998) found a group of isolates morphologically similar to *B. bassiana* which formed a genetically distinct group. Although, discrimination between disparate species and isolates of *Beauveria* is out of our research aims, identification of even one isolate necessitates a wide knowledge of species and their isolates.

Finally, isolate selection of entomopathogenic fungi especially *B. bassiana* for insect control is the aim of researchers to find the best isolates for mass production of them in the biological control programmes of insect pests. The first step in the introduction of the new isolates is their identification and further studies on their other characteristics such as virulence, cuticle-degrading enzyme production, toxin production, susceptibility to environmental conditions, stability, potential for mass production, effect on non-target organisms and etc. Morphologic characteristics including spore size and shape and colony form were used in this study as the first step in fungus identification. Further work was carried out with investigation on the presence of a well known gene *pr1* that there is in all defined isolates of *B. bassiana*. Nearly 70 per cent of insect integument has made of proteins (Charnley and St. Leger 1991). The chymoelastase Pr1 is a cuticle-degrading protease and determinant of pathogenicity (Paterson *et al*. 1994). This key enzyme has been detected in various isolates of *B. bassiana* (St. Leger *et al*. 1986a, b; Bidochka and Khachatourians 1990; Gupta *et al*. 1992; Shimizu *et al*. 1993) and purified from some isolates (Bidochka and Khachatourians 1987; Urtz and Rice 2000; Chrzanowska *et al*. 2001). Amplification of this gene was an emphasis for morphologic characteristics of the new isolate of *B. bassiana*. In the same way, ability of fungus for causing infection in host insect and recovering the fungus from dead insects showed again that this fungus was an entomopathogen and cause of death. Isolate characteristics such as stability, host range and cuticle-degrading enzymes profile should be considered in the coming works for its potential for application as mycoinsecticide.

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POLISH SUMMARY

IZOLACJA, IDENTYFIKACJA I OKREŚLENIE NOWEGO IZOLATU ENTOMOPATOGENICZNEGO GRZYBA *BOUVERIA BASSIANA* W IRANIE

Nowy izolat (BEH) wyosobniono z ziemi wykorzystując selektywną pożywkę DOC2. Izolat ten charakteryzował się trzonkami konidialnymi składającymi się z gęstych skupień krótkich komórek konidioforów z jednokomórkowymi, sferycznymi konidiami. Długość i szerokość konidiów wynosiła odpowiednio – 2,27±0,22 μm i 1,85±0,32 μm, przy stosunku długości do szerokości 1,23. Kolonie na pożywce SDYA pierwotnie były barwy od białej do jasno-żółtej czasem od spodu były zabarwione na czerwono.

Z powodu ważności tego patogena w programach biologicznego zwalczania i trudności z morfologiczną identyfikacją, opracowano technikę molekularną w celu uzupełnienia procedury określenia grzyba. Wykorzystując technikę PCR amplifikowano związaną z patogenicznością alkaliczną protezę seryny Pr1 degradującą kutikulę (skórkę), z określoną sekwencją w *B. bassiana*. Obecność tego genu w wyosobnionym grzybie (BEH) z sekwencją o długości 744 bp, potwierdziła dane uzyskane w toku badań morfologicznych – nowy izobat należy do entomopatogenicznego gatunku *B. bassiana*. Patogeniczność nowego izolatu dla Tenebrio monitor i jego reizolacja jest także potwierdzeniem przynależności do *B. bassiana*, co było zgodne z badaniem mikroskopem świetlnym.