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Identification and molecular characterization of an isolate of entomopathogenic fungus *Beauveria bassiana* from Meerut (UP, India) using RAPD-PCR

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Abstract
Molecular characterization in present work involved slight modification in standard CTAB method and ommision of poly vinyl pyrrolidone (PVP). Genetic variability present among different strains of *B. bassiana* can be studied through different PCR based molecular markers such as RAPD (Random Amplified Polymorphic DNA). A Known isolate, MTCC 4492 from (Institute of Microbial Technology), Chandigarh was included as standard in the experiments. Microscopic studies revealed presence of conidiophores consisting of whorls and dense cluster of short and globose cells with apical zig-zag appearance and one celled spherical conidia. Fungal colonies on SDYA media were white to pale yellow; some were red pigmented in reverse. Eight RAPD primers were used to characterize the genetic diversity present among isolates; they showed a total of 96 reproducible bands. Each of the primer varied greatly in their ability to resolve variability among the genotypes. The individual primer produced bands in a range of nine (LC103) to 18 (LC-89) bands with an average of 9.6 bands per primer. Out of the 96 bands, 1 band was monomorphic i.e. they were present in both isolates. The amplified fragment ranged from 280bp (primer LC-72) to 4000bp (primer LC-103). The polymorphism percentage ranged between 91.6% (primer LC-73) to 100%. The average polymorphic bands recorded as 9.5 bands per primer. Almost all primers based on 100% polymorphism and unique band amplification was considered as highly informative primers. Also the virulence of the fungus was observed with a control isolate to check its ability to cause infection of insect pest, cause its death and emerge out of its dead body.

Keywords: *Beauveria bassiana*, entomopathogenic fungus, CTAB, RAPD - PCR

Introduction
The entomopathogenic fungus *Beauveria bassiana* is a globally distributed Hypomycete, strain of which infect a wide range of insects. This fungus is a facultative saprophyte and there are reports of *B. bassiana* growing as a plant endophyte and interacting with plant roots (Wagner and Lewis, 2000; Lewis *et al*., 2001; White *et al*., 2002). The genus *Beauveria* describes a complex of morphologically similar and closely related species. So it is important to morphologically distinguish among various isolates of *Beauveria* (Glare and Innwood, 1998) [14]. 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 mostly spherical conidia and latter more cylindrical conidia (Brady, 1979a, b) [5-6]. Researchers have shown the change in spore shape after culturing (Townsend *et al*., 1995) [38]. To overcome these difficulties various molecular techniques were developed to assist the identification of *Beauveria* specie upto attained level (Kosir *et al*., 1991; St. Leger *et al*., 1992; Hegedes and Khachatourians, 1993 & 1996; Bidochka *et al*., 1994 ; Maurer *et al*., 1997) [23, 33, 19-20, 3, 25]. In culture, *Beauveria bassiana* grows as a white mold. Although a sexual stage is not known (Li *et al*., 2001) most *Beauveria bassiana* exsist in asexual reproducing form mainly through the production of single celled conidia. *B. bassiana* produces three single cell forms – aerial conidia, *in vitro* blastospores and submerged conidia in different conditions (Jeffes *et al*., 1999). Aerial conidia are produced on the surface of solid medium by a process of hyphal extension, formation of phialides (rachis) and spore production. These aerial conidia are used generally for biological control of agricultural pests because they are relatively resistant to varying environmental conditions and can be formulated to prolong shelf life. Aerial conidia contain a rodlet layer that results in a hydrophobic property (Holder and Keyhani, 2005). Blastospores are produced in liquid nutrient medium. They are hydrophilic, germinate and grow at a much higher rate than aerial conidia. Submerged conidia are produced in defined liquid medium are hydrophilic and show a rough surface morphology.

As aerial conidia function mostly as biocontrol agents, hence microscopic studies on them have revealed a spore ball structure of *B. bassiana* comprising of a cluster of short – globose to flask shaped conidiogenous cells which terminate in a narrow apical extension called "rachis". The rachis elongates after each conidium is produced resulting in a long zig – zag extension. The conidia are produced in an acropetal succession (youngest at the base and oldest at the tip).

Beauveria bassiana has been reported as a heterogenous assemblage of strains (Mugnai *et al*., 1989) [28]. Leger *et a*l. (1992) demostrated a high natural variability in population of B. bassiana in an extended isoenzyme analysis of 138 isolates broadly representing the reported range of geographical regime and hosts for the species. B. bassiana is a frequent candidate for biological control and so the diversity on various hosts in different geographical regions of the world need to be studied. Molecular approaches with isolating DNA and its analysis. Therefore, genetic variability present among different strains of B. bassiana can be studied through different PCR based molecular markers such as, RAPD, RFLP, SSR, ISSR and AFLP. The genetic structure and nucleotide sequences were reported for PCR amplification of RAPD primer binding sites, to establish the correct identification of entomopathogen *B. bassiana*. In the present study morphological measurements and simple molecular techniques along with assisting virulence of obtained fungal inoculums, just to check the ability of isolate to establish the disease and death in insect host were initiated to establish a base for further exper*iments.*

Materials and Methods

Isolation of fungus from soil

Generally, isolation of *B. bassiana* from soil requires a selective medium. DOC2 medium that contained no dextrose, containing 3g Bactopeptone,0.2g $CuCl_2$, 2mg crystal violet, 15g Agar and 1000 ml distilled water (Shimazu and Sato, 1996) was used for isolating fungus from soil samples (pH 10). The medium was autoclaved at 120ºC for 20 minutes and poured into 9 cm Petri plates. Soil sample (1g) from a sugarcane field, Sardar Vallabhbhai Patel University of Agriculture & Technology, Meerut (UP) was suspended in 200 ml of sterile distilled water containing 0.03% Tween ®80 as surfactant. Suspensions were applied at concentration of 0.2 ml/plate using Spread Plate Method. Plates were incubated at 25ºC in complete darkness. Obtained colonies were transferred to Sabouraud's Dextrose Agar Plates supplemented with 1% Yeast extract (SDYA) for primary morphological identification. Regular sub culturing of fungal colonies on SDYA plates maintained fungal inoculums for further experiments.

Extraction of DNA

A Known isolate, MTCC 4492 from (Institute of Microbial Technology), Chandigarh was included as standard in the experiments. SDB (Sabouraud's Dextrose Broth) medium consisting of 2% glucose, 0.5% peptone, 0.5% yeast extract was used as liquid culture for fungal growth. 100 ml conical flask containing 50 ml sterile SDB medium were inoculated with conidia from 14 day old SDYA culture and incubated at 120 rpm in an orbital shaker at 27ºC. For DNA extraction standard CTAB (cetyl trimethyl ammonium bromide) protocol was used with certain modifications. After 5 days mycelium was harvested using filtration by No.1 Whatman filter paper. Harvested mycelium was ground to fine powder in chilled pistil and mortar using liquid Nitrogen and ethanol sterilized cover slips for effective disruption of fungal cell wall and cell membrane. Powder was suspended in 10 ml of DNA extraction buffer (Tris Buffer (50 mM), EDTA (100 mM), NaCl (150 mM)). After proper shaking 1 ml of 10% SDS was added. The mixture was shaken gently at 37ºC for 1 h.1.5 ml of 5 M NaCl was added and mixed gently but thoroughly. 1.25 ml of CTAB/ NaCl solution was added, mixed thoroughly and incubated at 65ºC for 20 minutes in a incubator shaker at 60 rev per min. DNA was extracted by adding an equal volume of Chloroform: Isoamyl Alcohol (24: 1, v/v) and mixed thoroughly but gently, then centrifuged at 10000 rpm for 12 min at 10ºC. Aqueous viscous supernatant was removed to a fresh tube and precipitated with 0.6 volume of ice cold isopropanol and 0.1 volume sodium acetate and left overnight in the freezer at -20^{0}C. The mixture was centrifuged at 10000 rpm for 10 min at 10^{0}C. Pellet was washed with 70% ethanol and then dried completely. Pellet was then dissolved in minimum amount of TE buffer.

DNA pellet was purified further 2 µl RNAse A was added to the ependorf tube containing 200 µl of extracted DNA and then incubated for 3 h at 37ºC in a water bath. The DNA was further extracted with equal volume of Phenol: Chloroform: Isoamyl alcohol (25 : 24 : 1, v/v) and centrifuged at 10,000 rpm for 12 min at 10ºC. Supernatant was taken into a fresh eppendorf tube. 0.6 volume ice cold isopropanol and 0.1 volume of ice cold sodium acetate (3 M) were added and the mixture was kept at -20^{0}C for at least 2 h. The mixture was then centrifuged at 10,000 rpm at 10ºC for 12 min. Supernatant was removed using a micropipette and pellet was washed with 70% ethanol and dried completely. The DNA pellet was redissolved in minimum amount of TE buffer. Extracted DNA was qualified by Agarose Gel Electrophoresis (1.5% Agarose in 1XTAE buffer (prepared with 1:9 dilution of 10X TAE buffer-48.4g tris base, 11.4 ml glacial acetic acid, 3.7g EDTA for 1000ml buffer) stained with 5µl ethidium bromide (Genei). 6µl of extracted DNA was mixed with tiny drop of DNA loading Buffer in wells along with 1kb ladder DNA and subjected to constant voltage of 50V for 80 minutes.

Primers and PCR Amplifications

Polymerase chain reaction (PCR) Amplification, a molecular technique to create multiple copies of DNA without using a living organism was performed with primers obtained from Bangalore Genei, India. A set of 8 RAPD primers was subjected to amplify the DNA of *B. bassiana* isolate from Meerut (UP) and of MTCC 4492 (IMTECH, Chandigarh). The list of primers is given in following table:

Table 1: RAPD primers and their codes used for PCR amplification of B. bassiana

Primers No.	Code used in present study	Primers Sequence	GC (%)
Primer-1	LC-72	AGTCAGCCAG	60
Primer-2	LC-73	AATCGGGCTG	60
Primer-3	LC-76	GTGACGTAGG	60
Primer-4	LC-78	GTGATCGCAG	60
Primer-5	LC-89	AGTCAGCCAC	60
Primer-6	LC-90	GTGAGGCGTC	70
Primer-7	LC-101	GGGGTGACGA	70
Primer-8	LC-103	AGGGCGTAAG	60

PCR reaction mixture (20µl) consisted of 1µl of DNA template (50ng/µl), 0.2µl of dNTPs mix (100 mM each), 0.3µl of Taq DNA polymerase (5U/ µl), 2.5µl of reaction

buffer (10X with $MgCl_2$), 1 µl of Primer (30 ng/ µl) and 15 µl of Deionized water. The Bio-Rad Thermo cycler was programmed as follows: 5 min at 94ºC for initial denaturation, 35 cycles of Denaturation for 1 min at 94ºC, annealing for 1 min at 42ºC, extension for 2 min at 72ºC and a final extension for 7 min at 72ºC.

Virulence of fungal inoculum

As *Beauveria bassiana* is an entomopathogen, the ability of fungal isolates to cause infection and disease in insects, recovering of fungus from dead insect cadaver, again morphological studies to look for the spore characteristics were used as another parameters in completing the identification process. MTCC 4492 and isolate from Meerut were used in this bioassay. Fully sporulated 14-day old SDYA Petri plates were selected for fungal inoculums preparation. Conidia were scrapped from plates in sterile conditions and transferred to 100 ml sterile flask. 10 ml of sterile distilled water containing 0.03% Tween® 80 was added to each flask as surfactant and gently agitated for 15 min. Concentration of conidia (10^8 conidia/ml) was estimated using Haemocytometer and 10 larvae white grub were dipped in suspensions for 30 sec. control larvae was treated with distilled water suspension. Batches of 5 larvae were placed in Petri plates covered with wet No.1 Whatman filter paper. Experiment was repeated twice and Petri plates were incubated at 27ºC. Mortality of larvae was recorded for 10 days and dead larvae were removed and placed in new Petri Plate covered with wet No.1 Whatman filter paper for emergence of fungus. Conidia from dead larvae were transferred into fresh SDYA Plates. Light microscopic studies revealed the spore shape and characteristics resembling to *B. bassiana*.

Results

There were approx two to five small colonies on DOC2 medium that resembled *Beauveria bassiana* transferred colonies on SDYA Plates showed primarily that fungus may belong to group of *Beauveria bassiana*. Single spore colony was made to prevent any variation in the colonies of fungus. Microscopic studies revealed presence of conidiophores consisting of whorls and dense cluster of short and globose cells with apical zig-zag appearance and one celled spherical conidia. Fungal colonies on SDYA media were white to pale yellow, some were red pigmented in reverse. The extracted DNA was used for amplification with defined primers. The result of RAPD-PCR on extracted DNA of MTCC 4492 (as control isolate) and Meerut isolate are shown in figure.1 and figure.2, respectively.

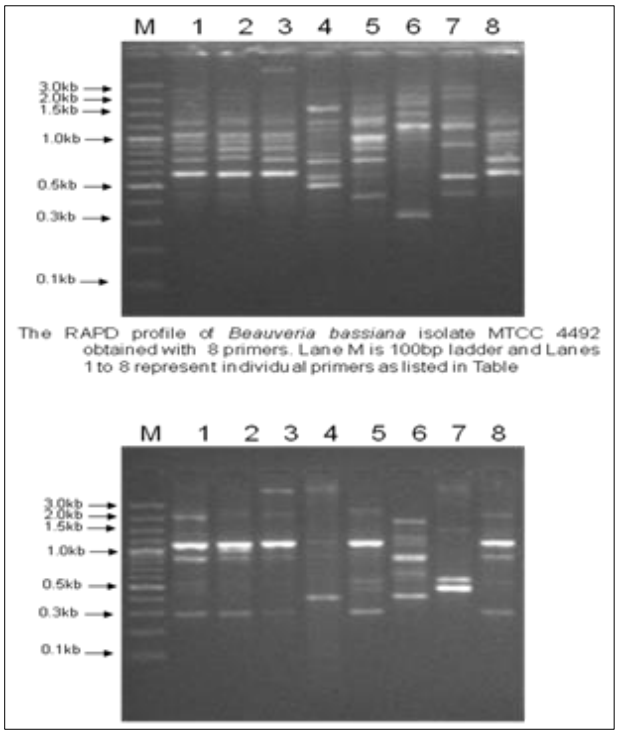

Fig 4.6: The RAPD profiles of *Beauveria bassiana* isolate obtained with 8 primes. Lane M is 100bp ladder and Lanes 1 to 8 represent in dividual primers as listed in table

Table 2: Details of RAPD primers used for the molecular characterization of isolates of *Beauveria bassiana*

S. No.	Primer code	Primer Sequence	Amplified product range (bp)	Total bands	Mono bands	Poly bands	% Poly.
1	LC-72	AGTCAGCCAG	280-2100	10	-	10	100.0
2	LC-73	AATCGGGCTG	480-3900	12	1	11	91.60
3	LC-76	GTGACGTAGG	520-3000	13	-	13	100.0
4	LC-78	GTGATCGCAG	330-3700	10	-	10	100.0
5	LC-89	AGTCAGCCAC	300-3800	18	-	18	100.0
6	LC-90	GTGAGGCGTC	290-3600	10	-	10	100.0
7	LC-101	GGGGTGACGA	280-3700	14	-	14	100.0
8	LC-103	AGGGCGTAAG	400-4000	9	-	9	100.0
Total				96	1	95	791.6
Average				9.6	0.1	9.5	79.16

Eight RAPD primers were used to characterize the genetic diversity present among isolates, they showed a total of 96 reproducible bands. Each of the primer varied greatly in their ability to resolve variability among the genotypes. The individual primer produced bands in a range of nine (LC103) to 18 (LC-89) bands with an average of 9.6 bands per primer. Out of the 96 bands, 1 bands was monomorphic i.e. they were present in both isolates. The amplified fragment ranged from 280bp (primer LC-72) to 4000bp (primer LC-103). The polymorphism percentage ranged between 91.6% (primer LC-73) to 100%. The average polymorphic bands recorded as 9.5 bands per primer. Almost all primers based on 100% polymorphism and unique band amplification was considered as highly informative primers. PCR product results showed a similarity between the genetic structure of MTCC 4492 and Meerut isolate. Results of fungal virulence towards host insect for MTCC 4492 and Meerut isolate were compared at subsequent intervals and morphological characteristics of both fungus arising from dead larvae were observed with resembling of spore characters. Although virulence of Meerut isolate on larvae was calculated low in comparison to MTCC 4492, yet it had ability to kill larvae and emerge out of its body and produce aerial mycelia and conidia on it. This emergence showed that the isolate is an entomopathogen

which caused larvae to die.

Discussion

The ability of *Beauveria bassiana* to grow in adverse conditions such as different pH range, poor nutrient availability makes DOC2 medium a potential medium for fungus isolation. This entomopathogenic fungus has the capacity to grow in a wide range of pH from 4 to 11 (Shimazu and Sato, 1996) [31]. High pH along with addition of $CuCl_2$ inhibited the growth of Penicillium spp. But did not prevent the germination and development of *Beauveria bassiana* on DOC2 medium. The use of selective medium and growth of fungus in an alkaline medium hence emphasized on results of Shimazu and Sato (1996) [31] for high ability of fungus to grow in adverse and severe conditions.

Morphological characteristics described here were in agreement with those have been defined by Samson *et al*., (1998) [30]. Although morphologic criteria are the necessary characteristics for intespecific and intraspecific discrimination among organisms, other parameters are also needed to be focused on when identifying an organism with confirmation. The genome of *Beauveria bassiana* has been determined to be 34.3-44.1 MB (Viaud *et al*., 1996) [40]. This large weight of DNA can be the cause of precipitation 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 specially annealing temperature of primers is the crucial step in the success of PCR amplification. These were calculated and set in Bio-Rad thermo cycler. PCR amplifications showed a similarity between MTCC 4492 and Meerut isolate of B.bassiana when amplified with 08 RAPD Primers. Due to its importance in biocontrol programs worldwide, B.bassiana, a number of techniques have been used in its identification (Glare and Innwood, 1998) [14]. Mugnai *et al*., 1989 [28] used biochemical and morphological testing for separating Beauveria species. In most tests *Beauveria bassiana* produced both spherical and ellipsoidal conidia. They concluded that spore size was variable in vitro but ellipsoidal conidia can be produced on the host, but only spherical conidia in the culture (Townsend *et al*., 1995; Glare and Innwood, 1998) [38, 14].

Finally, isolate selection of entomopathogenic fungi especially B.bassiana for insect control is the aim of researchers, to find the best isolate for mass production and its usage in biological control programmes. After identification it becomes mandatory for researchers to look onto other parameters such as virulence, toxin production, cuticle degrading enzyme production, susceptibility to environmental conditions, stability, potential for mass production and effect on non-target organisms. In the present study work, primarily identification was carried out by observing various characters such as spore shape and size, colony form, colour of colonies etc. Further amplification of DNA using RAPD primers for PCR with comparison along a known isolate provided access in identifying the fungus specie. In the same way, ability of the fungus for infecting an insect, causing its death and emerging out of its dead body showed again that this fungus was an entomopathogen. This work provided an isolate that has the virulence capacities which can be used for better mass production and use in further experimental research works for biocontrol programmes.

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