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Research Article

PCR based amplification of chitinase gene from native isolates of *Beauveria bassiana*

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ABSTRACT: Chitinase gene has been widely studied as one of the pest control agents because of its characteristic of degrading chitin. Chitinase is found in insects, nematodes, fungi, some algae and yeast. *Beauveria bassiana* is an insect pathogenic fungus successfully used as an insect pest control agent worldwide. In the present study, the entomopathogenic fungal isolates, *Beauveria* were isolated from the soil samples and insect cadavers collected from different places of Tamil Nadu. CTAB method was used for the isolation of DNA from entomopathogenic fungus. The ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and PN16 (5'-TCCCTTTCAACAATTTCACG-3') primers amplified a fragment of 930bp corresponding to the ITS1 and PN16 regions of the rDNA for the *Beauveria* isolates examined. Totally, 15 isolates were examined for the amplification of ITS region and all these isolates showed amplified product with size range of 930 bp which showed these isolates were belongs to *Beauveria* spp. A chitinase gene (1047 bp) was amplified from isolates of *B. bassiana* genomic DNA by PCR technique using forward primer 523Chit*Eco*RI (5'-ACATAGGAATTCATGGCTCCTTTTCTTCAAAC-3') and reverse primer 325Chit*Hind*III (5'-TACCTAACATGAACATTTAAGCTTTT-3'). All the isolates of *Beauveria* showed amplification at 1047bp. Chitinase has been considered as one of the most important factor for the pathogenicity of *B. bassiana* to various insect pests.

KEY WORDS: *Beauveria bassiana*, chitinase gene, Bbchit1 gene

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INTRODUCTION

Biological control agents are being considered as alternatives to synthetic chemical insecticides that are known to have toxic effects on non-target organisms, including animals and humans. Entomopathogenic fungi are key regulatory factors of insect populations in nature and are attracting attention as biocontrol agents for insect pests. Improvements in the virulence of entomopathogenic fungi can be achieved by understanding mechanisms of pathogenesis and genetically modifying targeted virulence factors. One of the advantages of entomopathogenic fungi is that the host insect need not ingest them. Entomopathogenic fungi, such as *Beauveria bassiana*, infect the host insect by penetrating the insect cuticle. The fungal conidium attaches to the cuticle by nonspecific hydrophobic mechanisms (Boucias *et al*., 1988). After cellulose, chitin is the most abundant polymer found in nature. The insect cuticle, the first barrier against fungal pathogens, consists of a thin outer epicuticle, containing lipid and proteins, and a thick procuticle, consisting of chitin and proteins (Clarkson *et al*., 1996).

Entomopathogenic fungi produce proteases, chitinases, and lipases which can degrade insect cuticle, allowing hyphal penetration through the cuticle and access to the nutrient rich insect hemolymph (Charnley and Leger, 1991). Chitinases are widely distributed and are found in viruses, bacteria, fungi, plants, and animals. Chitinase gene has been widely studied as one of the pest control agents because of its characteristic of degrading chitin, a chain homopolymer of N-acetylglucosamine (GlcNAc) connected by β-1,4 glucosidic linkages (Kramer *et al*., 1993). Overexpression of the *Bbchit1* gene from the entomopathogenic fungus *Beauveria bassiana* in transgenic *B. bassiana* can significantly enhance the virulence of *B. bassiana* for aphids (Fang *et al*., 2005). Here studies were made to identify the chitinase gene from *B. bassiana* isolates collected from different places of Tamil Nadu.

MATERIALS AND METHODS

Isolation of entomopathogenic fungal isolates from soil

Isolates of *B. bassiana* were obtained from soil using the bait method. Larvae of *H. armigera* and soil samples were collected from different tomato growing regions of Tamil Nadu. They were brought back to the laboratory and kept in a refrigerator before use. Each soil sample was placed in four separate plastic Petri dishes, 35 mm in diameter and a small quantity of sterilized water was added to each dish. Ten bait larvae were placed in each dish and the dishes were kept at room temperature (28 ± 2°C). Each

larva was removed after 24 h of burying, transferred to a test tube of 18 mm x 180 mm covered with cheese cloth and fed with a tomato leaf. Larvae were checked daily and cadavers were placed in 35-mm Petri dishes with moistened filter paper after 2-3 days of drying in the tube (Shimazu 1993). To isolate the *B. bassiana*, SDAY medium (Sabouraud's dextrose with 1% yeast extract agar medium) (barley flour 50 g; dextrose 10 g; neopeptone 4 g; yeast extract 2 g; agar 18 g; distilled water 1 L) was used. Conidia that formed on the cadavers were streaked onto SDAY. After incubation at room temperature (28 ± 2°C) for a week, the colonies obtained were transferred to SDAY slants for further studies. The fungal isolates were identified by microscopically inspecting the conidia forming mycelia for conidiogenous structures and conidial morphology (Samson *et al*., 1988)

DNA isolation

The cultures of *Beauveria* isolates maintained in SDY slants were transferred to SDY plates and incubated at 28°C for 2-4 days. All these were then transferred into 250 ml Erlenmeyer flasks containing 150 ml SDY broth and were incubated at room temperature for 7 days. Mycelium was harvested by filtration through sterile filter and stored at -70°C until used for DNA extraction. To extract the DNA, 1 g of frozen mycelium was ground to fine powder in liquid nitrogen and incubated in 5 ml, 2 per cent CTAB extraction buffer (10 mM trisbase (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, CTAB (2%), mercaptoethanol (0.1%) and PVP (0.2%) at 65°C for 1 h. The suspension was added with equal volume of phenol-chloroform-isoamylalcohol (25:24:1) mixture. It was vortexed to mix two phases, followed by a centrifuge at 12,000 rpm for 5 min. The supernatant was transferred to clean tube and mixed with equal volume of ice cold isopropanol. It was incubated at 25°C for DNA precipitation. The precipitate was collected by centrifugation and the pellet was washed with 0.1 M ammonium acetate in 70 per cent ethanol. Again incubation was given for 15 min. The pellet was resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and the DNA concentration was estimated spectrophotometrically (Zolan & Pukkila, 1996).

PCR amplification of ITS region

The reaction mix for PCR amplification of the DNA consisted of 20 µl vol, (0.25 mM each of primer pair, 0.25 mM dNTP, 1.5 mM $MgCl_2$, 50-80 ng of template DNA, 2 U of Taq DNA polymerase and 1x PCR buffer mix). To confirm strains as *B. bassiana,* intervening species specific sequence ITS1 (5'TCCGTAGGTGAACCTGCGG3'), PN16 (5'TCCCTTTCAACAATTTCACG3') primers were used to get an amplicon size of 930 bp. PCR was undertaken using a Mastercycler gradient (Eppendorf, Germany) using 0.5 µl tubes. Conditions were 2 min preheating step at 95°C followed by 35 cycles consisting of denaturing at 95°C for 1 min, 50°C annealing for 30 sec, extension at 72°C for 2 min and with a final extension at 70°C for 7 in (White *et al*., 1990). Amplification products were monitored by electrophoresis in 1.5% agarose gels, running 20 µl of amplification reaction using TBE Buffer 1x (89 mM tris borate – 2 mM EDTA pH 8.0) at 4 V cm^{-1} during 2 h. The gels were stained with ethidium bromide and visualized under UV light and photographed and documented with an AlphaImager (Alpha Innotech, California, USA).

PCR amplification of *Beauveria* chitinase (*Bbchit1*) gene

PCR reaction for the amplification of *Bbchit1* gene (1047bp) from genomic DNA was done in a 20 µl reaction mixture that included the following components: 10 µM each of primer pair (Forward Primer: 523Chit*Eco*RI – 5' ACATAGGAATTCATGGCTCCTTTTCTTCAAAC–3'; Reverse Primer: 325Chit*Hind*III 5'–TACCTAACATGAACATTTAAGCTTTT–3'), 0.25 mM dNTP, 1.5 mM $MgCl_2$, 50-80 ng of template DNA, 2 U of Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India) and 1X PCR reaction buffer. PCR was performed in Mastercycler gradient (Eppendorf, Germany) using 0.2 ml thin walled PCR tubes. PCR amplification condition included 5 min preheating step at 95°C followed by 35 cycles consisting of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 2 min and followed by a final extension at 72°C for 10 min (Khemika *et al*., 2006). The PCR amplified DNA products were separated in a 0.8 per cent agarose gel at 75 V and documented using AlphaImager (Alpha Innotech, California, and USA).

RESULT AND DISCUSSION

Isolation of entomopathogenic fungi

The entomopathogenic fungal isolates, *B. bassiana* were isolated from the soil samples and insect cadavers collected from different places. In Tamil Nadu thirteen *B. bassiana* isolates were isolated from different districts namely; Coimbatore, Theni, Nilagiri, Madurai, Thirunelvelli, Tuticorin and two isolates were isolated from Kohima (Nagaland) and Chirapunji (Mehalaya). (Table 1). The fungus was identified based on the conidial structure observed under microscope. The colony growth ranged from dull white to pure white in colour. The fungi produced millions of conidia, which were hyaline, globose and single celled. Isolation of these entomopathogenic fungal pathogens from the soil and insect was previously studied by several authors. *B. bassiana* is a soil borne fungus observed to be pathogenic to many insects worldwide (Groden, 1989). Soil conditions such as high relative humidity, low temperature and

Table 1. Isolates of *Beauveria bassiana* collected from different places

S.No	Isolates	Place	Source
1	B1	Coimbatore	Soil
2	B2	Arachalore	Insect
3	B3	Madurai	Soil
4	B6	Agasthiar Hills	Soil
5	B9	Cumbum	Soil
6	B13	Periyar reserve	Insect
7	B20	Chirapunji	Insect
8	B21	Ooty	Insect
9	B22	Kovilpatti	Soil
10	B23	Coimbatore	Insect
11	B24	Periyakulam	Soil
12	B25	Kohima	Soil
13	B26	Udumalai	Insect
14	B27	Pollachi	Soil
15	B28	Theni	Soil

protection from solar radiation make an optimal environment for the survival and persistence of this fungal pathogens for several years (Gaugler *et al.,* 1989). Many strains of entomopathogenic fungi *viz*., *B. bassiana* and *Metarhizium anisopliae* have been isolated from the soil and insects of various crops in different countries by several workers (Kulkarni *et al*., 2008; Thakur and Sandhu, 2009) and tested against several insect pests (Wraight *et al*., 2010).

DNA Extraction, Purification and Quantification

The DNA pellets were white, thick thread like mass. This DNA obtained was further quantified by spectrophotometry and agarose gel electrophoresis. It was observed that *Beauveria bassiana* DNA fragments were observed to emit orange fluoroscence under UV lamp. The A260/A280 ratio for isolates of *Beauveria bassiana* DNA was found to be ranging from 1.7 to 2.1 spectrophotometrically. This study revealed the method adopted for extraction; purification and quantification of DNA were found to be suitable for molecular studies of *B. bassiana.*

PCR amplification of ITS region

The ITS1 and PN16 primers amplified a fragment of 930bp corresponding to the ITS1 and PN16 regions of the rDNA for the *Beauveria* isolates examined. Totally, 15 isolates were examined for the amplification of ITS region and all these isolates showed amplified product with size range of 930bp (Fig 1). The 930 bp ITS fragment amplified in this experiment matches the reports by Neuveglise et al. (1994), that permitted the differentiation of *Paecilomyces* from *Beauveria*. Similarly, Gaitan et al. (2002) reported that, primers ITS1 and PN16 amplified a 930 bp fragment for 95 isolates of *B. bassiana* and the isolate of *B. brongniartii*.

PCR amplification of *Beauveria* chitinase (*Bbchit1*) gene

Chitin is an important component of insect cuticle, which is the primary barrier against pathogens. Overexpression of Bbchit1 was able to increase the ablility of *B. bassiana* to digest insect cuticle, resulting in increased virulence against insects (Murad *et al*., 2007). Chitinases cleave the internal β-1, 4-N-acetyl-D-glucosamine linkages in chitin polymers. These enzymes may play a multipurpose role in the biology of insect mycopathogens. In present study, the presence of chitinase producing gene was identified by the PCR analysis using gene specific primer namely, forward primer 523Chit*Eco*RI, Reverse primer 325Chit*Hind*III used for the detection chitinase gene (*Bbchit1*). Almost all the isolates of *B. bassiana* showed amplification at 1047bp which indicates all the isolates are contain chitinase producing gene (Fig 2). This result is

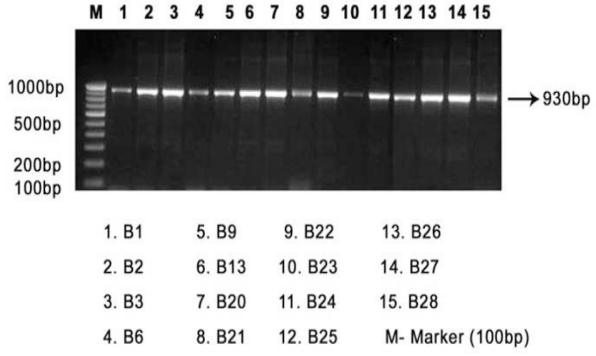

Fig. 1. PCR amplification of ITS fragments of *Beauveria bassiana* isolates

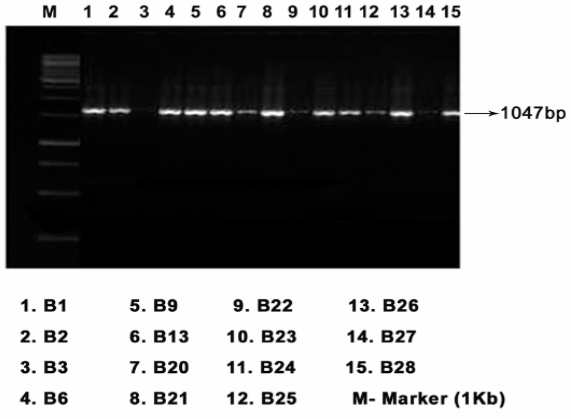

Fig. 2. PCR amplification of chitinase (Bbchit1) gene from *Beauveria bassiana*

further supported by Pallavi (2004), who observed that the entomopathogenic fungal pathogens produced extracellular chitinases against *H. armigera*.

REFERENCES

Fang W, Leng B, Xiao Y, Jin J, Ma Y, Fan J, Feng X, Yang Y, Pei Y. 2005. Cloning of *Beauveria bassiana* chitinase gene Bb*chit1* and its application to improve fungal strain virulence. *Appl Environ Microbiol.* **71:** 363–370.

Clarkson JM, Charnley AK. 1996. New insights into the mechanisms of fungal pathogenesis in insects. *Trends Microbiol.* **4:** 197–203.

Boucias DG, Pendland JC, Latge JP. 1988. Nonspecific factors involved in attachment of entomopathogenic deuteromycetes to host insect cuticle. *Appl Environ Microbiol*. **54:** 1795–1805.

Kramer KJ, Corpuz L, Choi HK, Muthukrishnan S. 1993. Sequence of a cDNA and expression of the gene encoding epidermal and gut chitinase of *Manduca sexta*, *Insect Biochem MolBiol.* 1993; **23**: 691-701.

Charnley AK, St. Leger RJ. 1991. The role of cuticle-degrading enzymes in fungal pathogenesis in insects, p. 267–287. *In* E. T. Cole and H. C. Hoch (ed.), *Fungal spore disease initiation in plants and animals*. Plenum Press, New York, NY.

Shimazu M. 1993. Isolation, culturing and preservation of entomogenous fungi. In: *Research Methods for Entomopathogens* (Eds. Okada, *et al*.), Japan Plant Protection Association, Tokyo, pp. 192-222.

Samson RA, Evans HC. Latge JP. 1988. *Atlas of Entomopathogenic Fungi*. Springer Verlag, Berlin. p.11.

Zolan ME, Pukkila PJ. 1986. Inheritance of DNA methylation in *Coprinus cinereus*. *Molecular and Cell Biol.* **6:** 195–200.

White TJ, Bruns T, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: a guide to methods and applications* (Eds. Innis MA, Gelfand DH, Sninsky JJ. White TJ.), Academic Press, San Diego. pp. 315–322.

Khemika S, Tawee D, Paranya C, Christopher R, Meyer. 2006. Cloning and Expression of Chitinase Gene isolated from insect pathogenic fungi, *Beauveria bassiana* in *Escherichia coli*. *Chiang Mai J Sci.* **33:** 347 – 355.

Groden E. 1989. Natural mortality of the colorado potato beetle, *Leptinotarsa decemlineata* (Say). *Ph.D Thesis*. Michigan State University, East Lansing.

Gaugler R, Costa SD, Lashomb J. 1989. Stability and efficacy of *Beauveria bassiana* soil inoculations. *Environ Entomol.* **18:** 412-417.

Kulkarni SA, Ghormade V, Kulkarni G, Kapoor M, Chavan SB, Rajendran A, Patil SK, Shouche Y, Deshpande MV. 2008. Comparison of *Metarhizium* isolates for biocontrol of *Helicoverpa armigera* (Lepidoptera: Noctuidae) in chickpea. *Biocont Sci Tech.* **18:** 809-828.

Thakur R, Sandhu SS. 2009. Distribution, occurrence and natural invertebrate hosts of indigenous entomopathogenic fungi of Central India. *Indian J Microbiol.* 1-8.

Wraight SP, Ramos ME, Avery PB, Jaronski ST, Vandenberg JD. 2010. Comparative virulence of *Beauveria bassiana* isolates against lepidopteran pests of vegetable crops. *J Inv Pathol.* **103**: 186–199.

Neuveglise C, Brygoo Y, Vercambre B, Riba G. 1994 Comparative analysis of molecular and biological characteristics of strains of *Beauveria brongniartii* isolated from insects. *Mycol Res.* **98:** 322–328.

Gaitan M, Valderrama, Saldarriaga, Velez, Bustillo. 2002. Genetic variability of *Beauveria bassiana* associated with the coffee berry borer *Hypothenemus hampei* and other insects. *Mycol Res.* **106:** 1307–1314.

Murad AM, Laumann RA, Mehta A, Noronha EF, Franco OL. 2007. Screening and secretomic analysis of entomopatogenic *Beauveria bassiana* isolates in response to cowpea weevil (*Callosobruchus maculatus*) exoskeleton. *Compar Biochem Physiol*. **145:** 333-338.

Pallavi BN. 2004. Development of biocontrol agents for the control of pests in agriculture using chitin metabolism as target. *Ph.D Thesis*. Division of Biochemical Sciences. National Chemical Laboratory. Pune. pp. 1-159.