Sequencing and Amplification of Chitinase Gene From Entomopathogenic Fungi Beauveria bassiana 6291

Shivani sood, Tapan Kumar Mukherjee*, SS Sandhu, Anil K Sharma

ABSTRACT--- The need for new and useful compounds to provide help and respite in all aspects of the human life is always growing. It has been well familiar that some plant pathogenic fungi can be developed as inundative biological control agents to kill or inhibit the activity of some insects which cause a large destruction in the crop fields and in forest vegetation. Today, scientist worked on a number of fungi for making it as a myco-insecticide because some fungi contain chitinase genes which degrade the outer hard cuticle of the insect pest. *Beauvaria bassiana* is one of the fungi which is widely used in insect-pest control in forest as well as in crop fields. In the present study, DNA of *Beauvaria bassiana* were extracted and amplified by PCR using designed primer from GeneRunner software for isolation of Bbchit1 gene. Bbchit1 gene showed significant similarities with *Cordyceps sp.*, *Metarhizium* , *Trichoderma* with endochitinase, chitinase and chit gene. The sequencing of the gene was done and subjected to ClustalX for alignment to detect conserved region and a phylogenetic tree was also generated. These results may be useful in characterizing the evolutionary mechanism of the species. The pathogenecity of the fungi was also detected against insect larvae of *Helicoverpa armigera* on the basis of time and temperature. This study will facilitate the identification of virulence genes and the development of improved bio-control strains with customized properties.

Index Terms:Entomopathogenic fungi, *Beauveria bassiana*, ClustalX, Pathogenecity, Phylogenetic tree, NCBI.

Introduction:

In the present scenario the main problem in crop fields and forest is to control the insect pest which causes a huge damage in this field. Due to vast use of chemical pesticides increase the resistant power in the insect. Now-a-day's biological control methods are the alternative pathway in controlling the pathogenic insect pest. A number of microorganisms like bacteria, fungi and virus are used to control the pathogenic organisms from the agriculture as well as from forest. Entomo-pathogenic fungi play an important role in shaping the larger context of insecticides with in contemporary insect pest management schemes [1,2,3].The entomopathogenic fungi were among the first organisms to be used as bio-pesticides. The term entomopathogenic fungi refer to fungi which induce disease symptoms in insect hosts. These fungi have very high potential as biocontrol agents against a number of insect pests. Entomopathogenic fungi such as *Verticillium lecanii*, *Beauveria bassiana* and *Metarhizium anisopliae, Nomuraea, Paecilomyces, Acremonium and Fusarium* are strongest natural enemies of the insect pest and hence are most commonly used mycobiocotrol agents [4,5] *Beauveria bassiana* is a globally distributed fungus which belongs to a class *Deutromycetes* and have dimorphic mode of life cycle[6,7].In the absence of the specific insect host these fungi show asexual vegetative life cycle. From *Beauveria bassiana* 16 products were formed by various companies which generally used in the crop fields of cabbage, potatoes, tomatoes, beans and also used for controlling the disease vectors like mosquitoes, flies and hematophagous insect pests. It has very high level of persistence and host specificity in the host population (*Glossina morsitans,Phlebotomus,Rhodnius, Hyblaeapara, Eutectina machaeralis*) and provide long term effect on pest suppression. *Beauveria bassiana* cause white muscardine disease in insect. When the spore of the fungi come in contact with the body of the insect they start germinate, penetrate into the cuticle and grow inside the insect which cause death of the organism within few days. Afterwards a white mold emerges from the cadaver and produce new spores [8]. Sarkar et al. [9] collected the 10 fungal strain of *B. bassiana* from the different sites of Pulney hill and tested the insecticidal activity of the fungi against the *Spodoptera litura* and found that it kills the insect at a range of 0.6-8.60%. Similarly Samuel et al. [10] reported the antibacterial activity of the *B. bassiana* against *H. hampei.* On the other hand Devi et al. [11] found that *B. bassiana* and *Nomuraea rieyi* showed 90% larvicidal activity against *H. armigera.* In the present study the

pathogenecity activity of the *B. bassiana* was observed and the gene chitinase which is responsible for their entompopathogenic activity was isolated and sequenced. The sequence analysis of chitinase gene(KF559204) submitted to NCBI and a comparative study was performed by using Clustal X(www.clustal.org/clustal2/) and phylogenetic tree was constructed by NJ method and UPGMA.

Material and Methods

Fungal Strains

In the present study the fungal culture of *Beauveria bassiana* (MTCC 6291 Strain E174) were taken from Institute of Microbial Technology, Chandigarh (India). The strain was reviewed and maintained on Potato Carrot Agar media and incubated at 25 ^{0}C for 5 to 7 days. A conidial suspension was also prepared and spread on the Yeast Extract Peptone Dextrose Agar (YEPD) plates and incubated at 25^{0}C. After 3 days conidia were collected from YEPD media and washed with sterile Tween 80 and filtered through a muslin cloth. A single spore and conidia were stored in 20% glycerol stock at -20^{0}C for further use.

Growth of *Beauveria bassiana*:

150 ml of Peptone yeast dextrose broth media was prepared in 250 ml flasks and autoclaved at 15 lbs psi for 20 min. The medium was inoculated with fungi culture and incubated at 25±1°C in the incubator. After one week of incubation a thick mat of the fungi was formed then it was dried and stored separately for DNA isolation.

Pathogenic activity of *Beauveria bassiana:*

Beauveria bassiana are potent entomogenous fungi in nature. They show activity against a number of insects like *Helicoverpa armigera,* chickpea borer etc. In this present work moths were collected from the different sites of agriculture fields with the help of light traps. The rearing of the insect larvae was done in glass jar on the chickpea leaves which was disinfected by sodium hypochlorite. The egg was daily sterilized by formaldehyde to prevent the viral and fungal infection. The new larvae comes out from the eggs were transferred on another normal diet required for growth and development of first instars. The pathogenic activity of the fungi depends upon various environmental factors viz. temperature, light, humidity and gases. In the present study, pathogenicity of *Beauveria bassiana* was carried out on *Helicoverpa armigera* wih respect to time and temperature. The conidia of *B.bassiana* was harvested from the 21 days old culture and their viability was observed as suggested by Gillespie [12]. The bioassay used to detect the effect of time and temperature on the activity of fungi was done as described by Sandhu et al. [4]. The conidial suspension of 5ml of fungi was sprayed on the test organism and calculated their LT_{50} after converting percent mortality into probits by regression analysis by Finney [13].

Extraction of fungal DNA:

The DNA was extracted by modified protocol of St. leger and Wang [14]. The mycelial mat of *Beauveria bassiana* was dip into the liquid nitrogen for few seconds and then crushed into the mortar with 0.7ml LETS buffer and 800 µl of phenol-chloroform isoamyl alcohol (PCI) was added and mixture was vortex for 5 minute. Then the mixture was centrifuged at 10000 rpm for 10 minute in cooling centrifuged. The upper aqueous layers were transferred into sterile micro-centrifuge tube and add 1ml chilled isopropanol to it. Spin the tube for 10 minutes at 10000 rpm and remove the supernatant and pellet was dried. Resuspened the pellet in 50 µl of nuclease free water for further study and the isolation of genomic DNA was confirmed by using agarose gel electrophoresis.

Amplification of chitinase Gene of *Beauveria bassiana* by Polymerase chain reaction:

For the amplification of desired gene Bbchit1, the specific primer (Table 1) were designed by Gene runner software and used to amplify the sequence of open reading frame (ORF) of gene. A reaction mixture of PCR was prepared by adding the desired chemicals in accurate concentration. Thirty cycles consisting of denaturation,annealing and extension were performed, then the product was checked on 1% agarose gel electrophoresis for specific band of desire gene.

Primers	Sequence
Forward	TTTCTTCAAACCAGCCTCGCGCT
Backward	AATGTCCAATTCTTGGAGCCGTCC

Table 1. Primers used for Polymerase Chain Reaction

Sequencing of the Gene:

The amino acid sequence was translated using ORF finder tool at NCBI. The submitted sequenced gene of chitinase was analyzed and aligned for homology similarity with the help of BLASTP tool [15] (www.ncbi.nlm.nih.gov/BLAST/). The sequence

showing maximum similarity was subjected to Clustal X(www.clustal.org/clustal2/) for multiple sequence alignment and generation of phylogenetic analysis by NJ and UPGMA method.(molbiol-tools.ca/Phylogeny.html.).

Results:

In the present study the fungal strain *B. bassiana* (MTCC6291 strain E174) was procured form Institute of Microbial Technology Chandigarh (India). The strain was revived and maintained on the Potato Carrot Agar for further study. For maximum production of fungi it was grown on peptone yeast dextrose broth at 28±1 ^{0}C. After one week of incubation a thick mat was formed this was separated by filter paper and preserved for DNA isolation. The pathogenicity test of *B.bassiana* was also carried against third instar larvae of *H.armigera.* to detect the percent mortality of insect larvae, conidial suspension *B. bassiana* was sprayed over 50 healthy larvae over different temperatures and with respect to time, observation were recorded as in Figure 1.

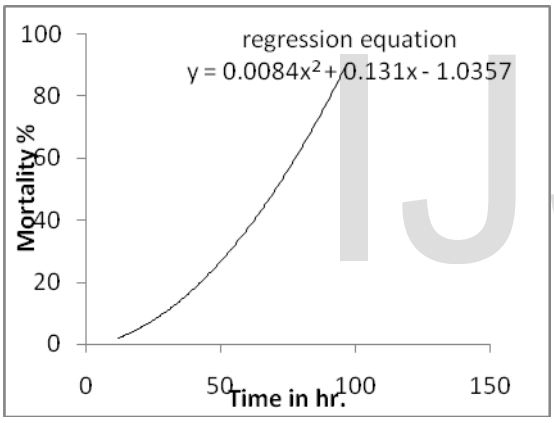

Figure 1. Virulence and Mortality of *B. bassiana* larvae at different time intervals

From the above figure we concluded that the virulence of *B. bassiana* increases with increase in time.The minimum virulence was shown at 12 hours (4%) and 24 hours (6%) whereas maximum virulence occured after 60 hours (35%), 72(60%) 84(72%) and in 96 (85%). Similarly the virulence of the fungi observed at different temperature like 15^{0}C, 20^{0}C, 25^{0}C, 28^{0}C, 30^{0}C, 35^{0}C and 40^{0}C. The fungus was most effective at 25^{0}C and 28^{0}C with a maximum larval mortality. But there was minimum mortality rate of larvae of *H. armigera* was detected as temperature increase as depicted in Figure 2.

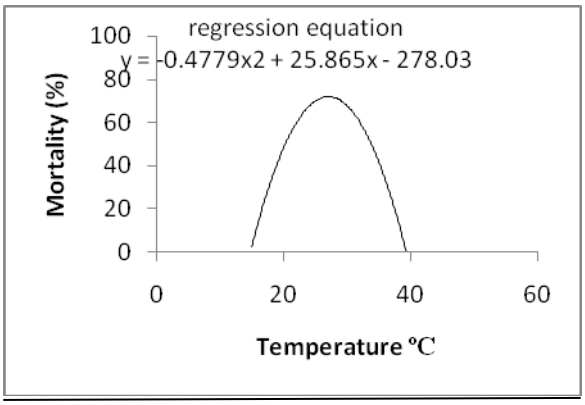

Figure 2. Virulence and Mortality of *B. bassiana* larvae at different temperature

Therefore, temperature and time period is the most important physical factors for maximum virulence of the *B.bassiana* fungi against insect larvae.After detection the virulence effects of the *B. bassiana* against *H. armigera* the DNA of the fungi was extracted by LETS method and conformed by using Agarose gel electrophoresis as well as quantified by spectrophotomatically (A260/A280). In Figure 3, the PCR reaction produced a size of 852 bp of DNA bands. No extra bands were detected suggesting that designed primers were specifically amplifying the chitinase gene fragment.

Sequence Similarity and Phylogenetic Analysis.

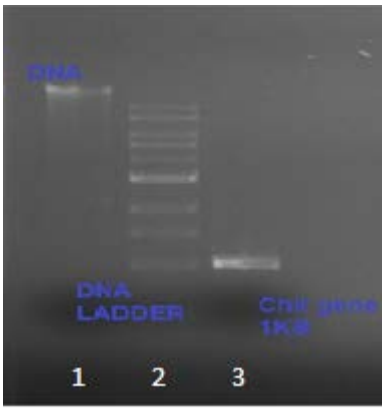

Figure 3 Agarose gel showing the amplified Bbchit1 gene (Well 1); Amplified PCR Product of Bbchit1(Well 3); DNA Ladder of 1 kb (Well 2)

The submitted sequence was translated to amino acids code by ORF finder tool available on NCBI, (http://www.ncbi.nlm.nih.gov/) which showed the sequence having no intron region. The sequence

obtained was subjected to homology search using BLASTP.

The sequence of the chitinase gene was deposited in NCBI Database as accession number KF559204 of 852 bp. The sequence is as follows*

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 12 atggtcggtgcctcaccgttagtcccccgagccgacacatgcgcg
    M  V  G  A  S  P  L  V  P  R  A  D  T  C  A
 57 accaagggaagacctacgggcaaggttctccaaggctattgggag
    T  K  G  R  P  T  G  K  V  L  Q  G  Y  W  E
102 aactgggacggttccagcaacggcgtgcatccgccctttggctgg
    N  W  D  G  S  S  N  G  V  H  P  P  F  G  W
147 acgccgatccagaaccccgacatccgcaagcacggctacaacgtc
    T  P  I  Q  N  P  D  I  R  K  H  G  Y  N  V
192 atcaacgccgccttcccggtcatcctgccggacggcacggcgctc
    I  N  A  A  F  P  V  I  L  P  D  G  T  A  L
237 tgggaggacggcatggacaccaacgtcaaagtcgccagcccggcc
    W  E  D  G  M  D  T  N  V  K  V  A  S  P  A
282 gacatgtgcgcggccaaggcggccggcgctaccatcctcatgtcc
    D  M  C  A  A  K  A  A  G  A  T  I  L  M  S
327 atcggcggcgcgactgccgccatcgacctgagctcctccgccgtc
    I  G  G  A  T  A  A  I  D  L  S  S  S  A  V
372 gctgacaagttcatcgctaccatcgtgcccattctgaaaaagtac
    A  D  K  F  I  A  T  I  V  P  I  L  K  K  Y
417 aactttgacggcatcgatatcgacatcgaggccggcctcacggga
    N  F  D  G  I  D  I  D  I  E  A  G  L  T  G
462 agcggcagcatcagcagcctgtccacgtcgcagaccaacctgatc
    S  G  S  I  S  S  L  S  T  S  Q  T  N  L  I
507 cgcatcatcgacggcatcctcgcgcagatgccgtccaactttggc
    R  I  I  D  G  I  L  A  Q  M  P  S  N  F  G
552 ctgaccatggcacccgagacggcctacgttaccggcggcagcgtc
    L  T  M  A  P  E  T  A  Y  V  T  G  G  S  V
597 acgtacggctccatctggggctcgtacctacccatcatcaagaag
    T  Y  G  S  I  W  G  S  Y  L  P  I  I  K  K
642 tacctcgacaacggccgtctctggtggctgaacatgcagtactac
    Y  L  D  N  G  R  L  W  W  L  N  M  Q  Y  Y
687 aacggcgccatgtacggctgcgcgggcgactcgtacgaggccggc
    N  G  A  M  Y  G  C  A  G  D  S  Y  E  A  G
732 accgtcaaaggcttcatggcccagacagactgcttgaacagcggg
    T  V  K  G  F  M  A  Q  T  D  C  L  N  S  G
777 ctgaccatccagggtgtgacgatcaagatcccctacgacaagcag
    L  T  I  Q  G  V  T  I  K  I  P  Y  D  K  Q
822 gttcctgggctgcccgctcagcctggcgcag 852
    V  P  G  L  P  A  Q  P  G  A
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*Nucleotide sequence of chitinase gene *Beauveria bassiana*, (GenBank accession number (KF559204). The deduced amino acid sequence is shown in one-letter code under the ORF.The sequence showed maximum similarity with *Cordyceps species (92%) and Trichoderma* species (85%). The Table 2 shows the accessions numbers and list of species which showed the homology similarities with chitinase gene. The sequences showing maximum similarity was subjected to multiple sequence alignment by CLUSTALX (Figure 4) and a phylogenetic tree was constructed based on the comparative analysis of related protein sequences using UPGMA method.

Accessions Numbers	Species
G3JH30	*Cordeyceps militaris*
K9JFD0	*Beauveria bassiana*
E5LEW9	*Beauveria bassiana*
Q8J1Y3	*Beauveria bassiana*
F6MIV5	*Beauveria bassiana*
D1MGZ8	*Beauveria bassiana*
E9DX57	*Metarhizium acridum*
D6N0Z5	*Trichoderma longibrachiatum*
A2VEC4	*Hypocrea jecorina*
D6N0Z4	*Trichoderma ghanense*
D6N0Z3	*Trichoderma citrinoviride*
C9WJD1	*Metarhizium anisopliae*
Q8NJQ4	*Trichoderma inhamatum*
Q8NJQ5	*Trichoderma harzianum*
C9WJDO	*Metarhizium anisopliae*
E9F7R6	*Metarhizium anisopliae*
A2SW11	*Bionectria ochroleuca*
D6N0Z1	*Trichoderma croceum*
G9MZY7	*Hypocrea virens*
Q2PPB2	*Trichoderma asperellum*

Table 2. Accessions numbersand species which showed the homology similarity with chitinase gene.

```
tr|Q8JIY3|Q8JIY3_DANRE      --------------------------------TVNASMSRFPPHMVPPHHSLHTTGIPHP
tr|Q2PPB2|Q2PPB2_9HYPO      TQVATPAEMCEAKAAGATILLSIGGATAGIDLSSSAVADKFIATIVPILKQYNFDGID--
tr|D6N0Z3|D6N0Z3_9HYPO      VKVATPAEMCQAKAAGATILMSIGGATAAIDLSSSTVADKFISTIVPILKQYNFDGID--
tr|A2VEC4|A2VEC4_HYPJE      VKVATPAEMCQAKAAGATILMSIGGATAAIDLSSSAVADKFISTIVPILKQYNFDGID--
tr|D6N0Z5|D6N0Z5_TRILO      VKVATPAEMCQAKAAGATILMSIGGATAAIDLSSSAVADKFISTIVPILKQYNFDGID--
tr|D6N0Z4|D6N0Z4_9HYPO      VKVATPAEMCQAKAAGATILMSIGGATAGIDLSSSAVADKFVSTIVPILKQYNFDGID--
tr|D6N0Z1|D6N0Z1_9HYPO      VQVATPAEMCQAKAAGATILMSIGGATAGIDLSSSTVADKFISTIVPILKQYNFDGID--
tr|G9MZY7|G9MZY7_HYPVG      VKVATPAEMCQAKAAGATILMSIGGATAGIDLSSSTVADKFVSTIVPILKQYNFDGID--
tr|Q8NJQ4|Q8NJQ4_9HYPO      VKVATPAEMCQAKAAGATILMSIGGATAGIDLSSSTVADKFISTIVPILKQYNFDGID--
tr|Q8NJQ5|Q8NJQ5_TRIHA      VKVATPAEMCQAKAAGATILMSIGGATAGIDLSSSTVADKFISTIVPILKQYNFDGID--
tr|A2SW11|A2SW11_BIOOC      VKVATPAEMCAAKAAGATILLSIGGATAGIDLSSSAVADKFVATVVPILKNYNFDGID--
tr|E9DX57|E9DX57_METAQ      VKVATPAEMCQAKAAGATILMSIGGAAAAIDLSSSTVADKFISTIVPILKKYNFDGVD--
tr|C9WJD1|C9WJD1_METAN      VKVATPAEMCQAKAAGATMIMSIGGAAAAIDLSSSSVADKFVSTIVPILKRYNFDGVD--
tr|C9WJD0|C9WJD0_METAN      VKVATPAEMCQAKAAGATMVMSIGGAAAAIDLSSSSVADKFVSTIVPILKRYNFDGVD--
tr|E9F7R6|E9F7R6_METAR      VKVATPAEMCQAKAAGATIVMSIGGATAAIDLSSSSVADKFVSTIVPILKRYNFDGVD--
tr|K9JFD0|K9JFD0_BEABA      VKVASPADMCEAKAAGATILMSIGGATAAIDLSSSAVADKFVSTIVPILKKYNFDGID--
tr|E5LEW9|E5LEW9_BEABA      VKVASPADMCEAKAAGATILMSIGGATAAIDLSSSAVADKFVSTIVPILKKYNFDGID--
tr|F6MIV5|F6MIV5_BEABA      VKVASPADMCEAKAAGATILMSIGGATAAIDLSSSAVADKFVSTIVPILKKYNFDGID--
tr|D1MGZ8|D1MGZ8_BEABA      VKVASPADMCEAKAAGATILMSIGGATAAIDLSSSAVADKFVSTIVPILKKYNFDGID--
seq1                        VKVASPADMCAAKAAGATILMSIGGATAAIDLSSSAVADKFIATIVPILKKYNFDGID--
tr|G3JH30|G3JH30_CORMM      VKVATPAEMCAAKAAGATILMSIGGATAAIDLSSAAVADKFIATIVPILKKYNFDGID--
                                                             :  :  .:*   :**  :  .  *:
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Figure 4. Multiple sequence alignment of chitinase sequences of Entomopathogenic Fungi in which * revealed highly conserved region whereas : revealed most identical region.

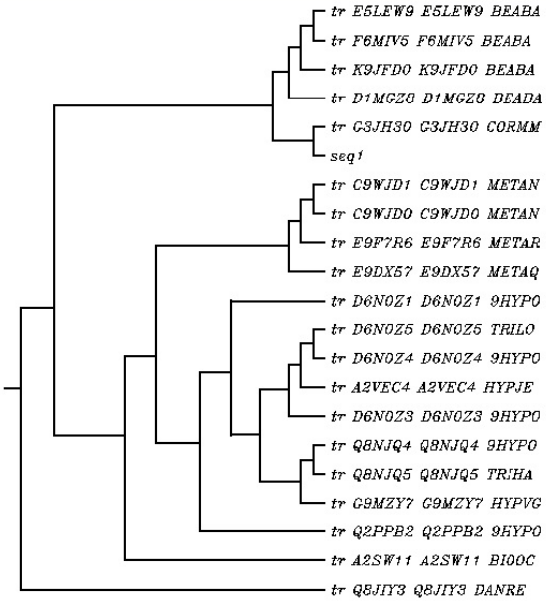

Figure 5. Phylogenetic analysis of Chitinase gene of *Beauveria bassiana* (seq1) with Entomopathogenic fungi which showed maximum evolutionary relationship with Cordeyceps militaris (G3JH30).

The Multiple sequence analysis reveals that these sequences contain highly conserved regions of amino acids as all organism share a region which is highly similar to chitinase gene of other entomopathogenic fungi. Similarly phylogenetic relationships (Figure 5) between the species of the entomopathogenic fungi were compared using multiple sequence analysis. Earlier *Bbchit1* had very low levels of identity to other chitinase genes when compare to previously isolated chitinase gene from entomopathogenic fungi, indicating that *Bbchit1* was a novel chitinase gene from an insect-pathogenic fungus [16]. Thus, it was reported the isolated chitinase is of great significance for improvement of its entomopathogenicity and could also provide a powerful tool for studying the functions of *B. bassiana* genes in general. In addition, experiments are needed to demonstrate that the changes in morphology, growth characteristics, and virulence observed with purified enzyme extract against pathogens.

Conclusion

All species of entomopathogenic fungi contains chitinase gene copy with highly conserved regions.The Multiple sequence analysis reveals that this sequence is highly conserved as all the organism share a region of similarity. In conclusion,sequencing of this important gene is the first step to pursue future research *in silico* sequence analysis.The sequence analysis of the gene fragment sequenced in this study is still under investigation. This analysis will help in future research to identify other species of entomopathogenic fungi.

References

[1] Lord JC (2005) From Metchnikoff to Monsanto and beyond: the path of microbial control. J Invert Pathol 89:19–29

[2] Roy HE, Steinkraus DC, Eilenberg J, Hajek AE, Pell JK (2006) Bizarre interactions and endgames: entomopathogenic fungi and their arthropod hosts. Annu Rev Entomol 51:331–357

[3] Khachatourians GG (2008) Insecticides, microbial. In: Schaecter M, Summers WC et al (eds) Encyclopedia of microbiology, vol 2, 3rd edn. Elsevier, New York

[4] Sandhu S. S., R.C. Rajak and Agarwal G. P. (1993a) – Annl. Of Forestry, Vol. 1 No 2: 136-140

[5] Roberts, D.W. and St.Leger, R.J. (2004). *Metarhizium* spp., cosmopolitan insect-pathogenic fungi: mycological aspects. Advances in Applied Microbiology, 54: 1– 70.

[6] De la Rosa W, Alatorre R, Barrera JF, Toreillo C. (2000). Effect of *Beauveria bassiana* and *Metarhizium anisopliae* upon the coffee berry borer (Coleoptera: Scolytidae) under field conditions. J Econ Entomol 93:1409–1414.

[7] Brownbridge M, Costa S, Jaronski ST. *(*2001). Effect of in vitro passage of *Beauveria bassiana* on virulence to *Bemisia argientifolii.* J Invertebr Pathol *77:280–283.*

[8] Sardul Singh Sandhu, Anil K. Sharma, Vikas Beniwal, Gunjan Goel, Priya Batra,Anil Kumar,Sundeep Jaglan, A. K. Sharma, and SonalMalhotra.(2012) Myco-Biocontrol of Insect Pests: Factors Involved,Mechanism, and Regulation Hindawi Publishing CorporationJournal of Pathogens Volume 2012, Article ID 126819, 10 pages doi:10.1155/2012/126819

[9] Sarkar, J., Ray, S., Chattopadhyay, D., Laskar, A., and Acharya, K. (2012) Mycogenesis of gold nanoparticles using a phytopathogen *Alternaria alternata*. *Bioprocess Biosyst Eng* 35: 637–643.

[10] Samuel, SD., S. Irulandi, G.A. Raj, P.K.V kumar and A. Ravikumar, 2009. Dyanmics of BB infecting Hypothenemus hampei and its management. Narosa Publishing house, new delhi, India , PP:62-67

[11] Devi, K.U., C.H.M. Mohan, J. Padmavathi and K. Ramesh, (2003). Susceptibility to fungi of cotton boll worms before and after a natural epizootic of the entomopathogenic fungus *Nomuraea rileyi* (Hyphomycetes). Biocontrol Sci. Technol., 13: 367-371.

[12] J.P. Gillespie, A.M. Bailey, B. Cobb, A. Vilcinskas(2002).Fungi as elicitors of insect immune responses Arch. Insect Biochem. Physiol., 44, pp. 49–68
[13] D.J. Finney(1952). Probit Analysis (second ed.)University Press, Cambridge (1952) 318pp
[14] St. Leger RJ, Wang C (2009) Entomopathonic Fungi and the Genomics Era. In: Patricia Stock SP, Vanderberg J, Boemare N, Glazer I, eds. Insect Pathogens: Molecular Approaches and Techniques, CABI. pp. 365-400.
[15] Altschul SF, Gish W, Miller W, Myers EW, Lipman D (1990) Basic local alignment search tool. J Mol Biol 215:403–410.
[16] Weiguo Fang, Bo Leng, Yuehua Xiao, Kai Jin, Jincheng Ma, Yanhua Fan,Jing Feng, Xingyong Yang, Yongjun Zhang, and Yan Pei (2004). Cloning of *Beauveria bassiana* Chitinase Gene *Bbchit1* and Its Application To Improve Fungal Strain virulence.PLoS One. 2013 Jul 5;8(7).
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molbiol-tools.ca/Phylogeny.htm
www.uniprot.org/