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Characterization and biocontrol potential of entomopathogenic fungus, *Beauveria bassiana* isolates against *Spilarctia obliqua*

B.P. Bhadauria[1]*, P.K. Singh[2], Shailesh Pandey[3] N.W. Zaidi[4] and U.S. Singh[4]

[1]*Department of Plant Pathology, G.B. Pant University of Agriculture and Technology, Pantnagar-263 145, India*
[2]*Department of Entomology, RBS College, Bichpuri, Dr. B. R. Ambedkar University, Agra-283 105, India*
[3] *Department of Forest Protection, Rain Forest Research Institute, Jorhat-785 001, India*
[4] *NASC Complex, IRRI Office, New Delhi-110 012, India*
Corresponding Author email : bpbhadauria@rediffmail.com

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Abstract

Beauveria bassiana is a known natural enemy of a number of insect pests of crop plants. In order to screen different isolates of any given entomopathogens molecular markers provide a means for constructing the molecular phylogeny, diversity and link to virulent phenotypes. Eight isolates of *B. bassiana* isolated from different insect hosts and from different location at Pantnagar (Uttrakhand) were characterized by PCR-based RAPD markers. Bioassays were conducted by using first, second and third instar larvae of *Spilarctia obliqua* in order to categorize the isolates based on virulence. The isolates were arbitrarily rated as more virulent, moderately virulent and less virulent based on the speed of killing. A wide range of variation in virulence was observed and the isolates of same insect origin and location showed differences in their aggressiveness. No correlation was found between the pathogenicity of the isolates and the relatedness of the original insect host. The pathogenicity against first, second and third instar larva of *Spilarctia obliqua* did not reveal any relatedness with the clustering pattern.

Key words

Beauveria bassiana, Biocontrol, DNA fingerprinting, *Spilarctia obliqua*, RAPD-PCR

Introduction

The indiscriminate use of chemical pesticides is assuming a serious cause of concern to human health and environment safety. An alternative to chemical pesticides in integrated pest management is always needed. The entomopathogenic fungus *Beauveria bassiana* is a promising and extensively researched biocontrol agent that can suppress a variety of economically important insect pests (Coates *et al*., 2002; McGuire *et al*., 2005). *B. bassiana* has a wide host range but differences in both host specificity and virulence among isolates has been reported (Devi *et al.,* 2008). However, it is increasingly being realized that this fungus is rather a generalist, with no strict host specificity (Rehner and Buckley, 2005). There are several *B. bassiana*-based mycoinsecticides currently registered or under commercial development for agricultural pests (Hajek *et al*., 2001). To determine its efficacy host specificity, survival and partial temporal distribution in the field, distinctive markers are needed for the individual strains (Leal *et al*., 1994). Bielikova *et al*. (2002) stated that the differentiation of fungal strains and species by RAPD is certainly an easy tool to detect polymorphisms in a large number of samples at relatively low cost. This technique utilizes short primers of arbitrary sequences that anneal to multiple target sequences, thus producing diagnostic patterns (Valderrama *et al*., 2000). *Spilarctia obliqua is* an important lepidopteron pest on a wide range of crops including tobacco, cotton and tomato throughout Europe, Asia, Africa and Australia (Anonymous, 2000). The pest has developed resistance to many of the conventional insecticides (Kranthi *et al*., 2002). In the present study laboratory bioassays with eight isolates

of *B. bassiana* against the first, second and third instar larvae of *Spilarctia obliqua* were carried out to identify virulent isolates. RAPD was also carried out with 10-mer operon primers to show polymorphisms among the isolates and among the primers

Materials and Methods

Fungus culture : Eight local isolates of *B. bassiana* (PBB-01 to BPP-08) were collected from different mango orchard areas of Govind Ballabh Pant University of Agriculture and Technology, Uttarakhand. The fungus was isolated from insect cadavers and their pure culture was maintained on Sabouraud Dextrose Agar (Dextrose Yeast Agar media Dextrose-4%; Peptone-1%and Yeast extract -1%) and PDA slant for further use. All the isolates were used for further pathogenecity studies. Isolate PBB-01 to BPP-02 has been identified from IMTECH Chandigarh with accession number: MTCC-9967 and MTCC 9968, respectively.

***In-vitro* bioassay :** The first, second and third instar larvae of *S. obliqua* were obtained by breeding the field collected insect in the laboratory. Female moths lay up to 300 eggs in masses which hatch within 3-5 days. The patches laid on castor leaves by the moths were carefully transferred onto fresh castor leaves for eggs to hatch. The neonate larvae were later transferred and maintained on fresh castor leaves until they reached second and third instar stage. Bioassays were done with these larvae and a homogenous population was maintained.

Newly moulted first, second and third instar larvae of *S.obliqua* were bioassayed for their susceptibility to *B. bassiana* isolates. Ten larvae were taken in a Petri dish which was lined with a filter paper at the bottom for absorbing excess moisture. Five different concentration viz. 10^5,10^6, 10^7, 10^8 and 10^9 conidia ml^{-1} were prepared in sterilized distilled water using serial dilution technique. The number of spores in each concentration was counted using a haemocytometer. Five ml of each of the different concentrations were then directly sprayed on the larvae using a hand automizer. Three replicates of ten larvae were used in each case. Same number of larvae sprayed with 5 ml of sterilized distilled water served as control. The larvae were air dried by keeping them in laminar air flow for 5 min and carefully transferred to individual clean sterile glass Petri plates containing fresh castor leaves. These plates were then kept inside the environmentally controlled growth chamber having 27±1ºC temperature and 75±5 relative humidity. The larval mortality was recorded at 24 hr interval until 9 days (216 hrs) of treatment. The percentage larval mortality due to mycosis was recorded and the results of assay were subjected to probit analysis using software STPR-18 for calculating the median lethal time (LT_{50}) and median lethal concentration (LC_{50})

DNA extraction and amplification : For DNA extraction, the isolates were grown in Sabouraud dextrose yeast peptone broth. Conidia from 7-day old pure slants of *B. bassiana* were scrapped and suspended in 0.02% Tween-80 solution, and the concentration was adjusted to 10^8 spores ml^{-1}. Conical flasks containing 75 ml of Sabouraud dextrose yeast peptone broth were inoculated with 2 ml of 10^8 spores ml^{-1} of conidial suspension and incubated in an rotary shaker (120 rpm) at 25ºC for a period of 3-4 days. The mycelium was harvested after 72 hr and washed 3-4 times with sterile distilled water and ground to a fine powder in liquid nitrogen. DNA was extracted according to the method of Lee and Taylor (1990) with slight modifications. Mycelia (500 mg) were ground in 5 ml of lysis buffer (50 mM Tris HCl, 50 mM EDTA, 3% SDS and 1% b - mercaptoethanol). The mixture was incubated at 65°C for 30 min. The mixture was centrifuged and an equal volume of phenol:chloroform (1:1) was added to the supernatant, mixed gently for 1 min and then centrifuged. This was done twice. To the aqueous phase, an equal volume of chloroform: isoamyl alcohol at a ratio of 24:1 was added, mixed gently and then centrifuged. To the recovered aqueous phase, 1/10th volume of sodium acetate (3 M) and an equal volume chilled isopropanol was added. After incubation at -20^{0}C for 30min, the DNA strings that appeared were recovered by centrifugation and washed with 70% ethanol. The DNA pellet was dried in vacuum for 20 min and the pellet was suspended in 50 – 100 µl of TE buffer (20 mM Tris HCl, 1 mM EDTA) or in sterile double distilled water, and stored at -20ºC until further use.

A total of 22 primers (Operon Technologies) were screened for amplification and polymorphism using a Perkin-Elmer Cetus Gene Amplification PCR system (Table 2) Model EP-Gradients serial no. 16842. The 25 ml reaction mixture consisted of 2.5 ml of PCR buffer (PCR buffer and 1.5 mM $MgCl_2$), 1.25 ml of 100 mM dNTP mixture, 0.5 ml of primer (10 mM),, 0.125 ml of *Taq* DNA polymerase, 1 ml of DNA (25 ng) and 19.625 ml of double distilled water. The selected temperature profile included initial denaturation for 3 min at 94ºC, denaturation for 40 sec at 94ºC, annealing for 40 sec at 37 ºC, extension for 1 min at 72°C and a final extension for 7 min at 72ºC for 30 cycles. The amplified PCR products were electrophoresed on a 1.5% agarose gel in TBE buffer. 100-bp ladder maker was used as a marker. The gels were stained with ethidium bromide and were photographed under UV illumination.

Statistical analysis : The data on bioassay was subjected to Probit Analysis (Finney, 1964) for calculating the regression lines, LT_{50} values and fiducial limits. Heterogeneity among the populations was performed by the Chi square test.

A binary matrix for presence or absence (1/0) of bands in the isolates with each primer was compiled. Based on the matrix, jaccard similarity cofficient (similarity values) between

all possible paired combinations of the isolates were calculated, as suggested by Sokal and Michener (1958). The similarity values were subjected to the sequential agglomerative hierarchical nested (SAHN) clustering using the unweighed pair group method arithmetic average; UPGMA employing NTsys- pc Version 2.1 to generate dendogram. Bootstrap values for the branches of the Phenogram were generated using Winboot program

Results and Discussion

The pathogenicity test which was carried out on first, second and third instar larvae of *S. oblique* showed differences in mortality rates among the eight isolates, studied. The least LT50 values of first instar larvae 130,135 and 140 hr. was recorded in isolates, PBB-01, PBB-2 and PBB-4 were thus categorized as highly virulent isolates (Table 1). The highest LT50 values of 157,162 and 164 hr were recorded in isolates PBB-07, PBB-05 and PBB-06. The isolates showing a range of LT50 from 146 to 145 hr. were categorized as moderately virulent, and two isolates were included in this category. The isolates which showed an LT50 values among 157,162 and 164 hr were categorized as less virulent, and three isolates showed this pattern. Second instar larvae of *S. obliqua*. The least LT50 values of second instar larvae 131 and 137 hr. was recorded in isolates PPP-1 and PBB-2 were thus categorized as highly virulent isolates. The highest LT50 values of 161,170 and 174 hr. were recorded in isolates PBB-5, PBB-6 and PBB-07. The isolates showing a range of LT50 from 146, 150 and 151 hr were categorized as moderately virulent, and three isolates were included in this category. The isolates which showed an LT50 value among 161,170 and 174hr were categorized as less virulent, and three isolates showed this pattern. Third instar larvae of *S. obliqua*. The least LT50 values of third instar larvae 142,148 and 153 hr was recorded in isolates PBB-1,PBB-2 and PBB-6 were thus categorized as highly virulent isolates. The highest LT50 values 164,161 and 169 hr were recorded in isolates PBB-4, PBB-5 and PBB-7. The isolates showing a range of LT50 from 159,161 and 156 hr were categorized as moderately virulent, and three isolates were included in this category. The isolates which showed an LT50 value between 164 and 169 hr were categorized as less virulent, and three isolates showed this pattern. Chi square test showed no heterogeneity among the populations tested. LT50 values showed a good fit and were well within the range of fiducial limits. The isolates that were more effective in the first, second and third instar were PBB-1, PBB-2 and PBB-6

For the success of any biocontrol program, isolation, characterization and selection of highly virulent and aggressive isolates is of utmost importance. Variation between isolates from single or multiple geographical locations and from different hosts necessitates the need for specific parameters for their identification. In the present study, a wide range of variation in virulence was observed and the isolates of same insect origin and location showed differences in their aggressiveness. There is no correlation between aggressiveness of the isolates and the relatedness of the original insect host. Isolates 2, 3 and 5 were obtained from mealy bugs but differed in their virulence. Such variations among *B. bassiana* isolates has been observed in bioassays with the coffee berry borer, sugarcane stem borer and the darkling beetle (Gurvinder *et al.,* 2008). Older larval stages of *S. oblique* was found less susceptible in comparison to early larval stages (Bhadauria *et al.,* 2011). An overall lack of relationship between the clustering pattern and pathogenicity and the original insect host from which the isolates were isolated was recorded. Isolates 1 and 2 found highly virulent but grouped into different clusters with less aggressive isolates. Luz *et al.* (1998) and Devi *et al.* (2001) found similar results with *B. bassiana* isolates tested against *Triatoma infestans* and sorghum shoot borer, respectively, the virulence of the isolates was not correlated with RAPD groups. Valderrama *et al*. (2000) found no correlation between the clusters obtained by RAPD analysis of the insect host and the pathogenicity of *Hypothenemus hampei* (Coffee berry borer).

Table 1 : Evaluation of *Beauveria bassiana* isolates for virulence against first, second and third instar of *S. obliqua*

Isolate	LT_{50} (hour)			Fiducial limits		
	1st instar	2nd instar	3rd instar	1st instar	2nd instar	3rd instar
PBB-01	130***	131***	142***	112.35 to 154.89	102.74 to 133.86	126.27 to 162.64
PBB-02	135***	137***	153***	119.23 to 156.77	107.98 to 140.42	135.51 to 178.33
PBB-03	146**	150***	159**	130.12 to 167.95	132.64 to 175.10	138.28 to 191.36
PBB-04	140***	146**	164*	123.12 to 164.75	127.68 to 173.52	141.50 to 201.80
PBB-05	162*	174*	161**	143.64 to 189.39	148.31 to 224.07	138.72 to 197.20
PBB-06	164*	161*	148***	143.05 to 198.90	136.71 to 203.78	130.77 to 173.63
PBB-07	157*	170*	169*	136.80 to 189.62	145.32 to 211.95	145.93 to 210.31
PBB-08	145**	151**	156**	126.12 to 173.73	132.62 to 178.56	133.64 to 193.19

*Less virulent **Moderate virulent *** High virulent

Table 2 : List of RAPD primers which showed amplification

S. No.	Primer details	Sequence
1	LC-71	TGCCGAGCTG
2	LC-72	AGTCAGCCAG
3	LC-73	AATCGGGCTG
4	LC-74	AGGGGTCTTG
5	LC-75	GAAACGGGTG
6	LC-76	GTGCAGTAGG
7	LC- 78	GTGATCGCAG
8	LC-80	CAGCACCCAC
9	LC-84	AGGTGACCTG
10	LC-89	AGTCAGCCAC
11	LC-91	TGGACCGGTG
12	LC-93	GGACCCAACC
13	LC-101	CTCTCCGCCA
14	LC-102	CATCCGTGCT
15	LC-103	AGGGCGTAAG
16	LC-104	GAGAGCCAAC
17	LC-105	ACCCGGTCAG
18	LC-110	GGACTGCAGA
19	LC-111	CTCTCCGCCA
20	LC-113	AGACGTCCAC
21	LC-114	GACGCCACAC
22	LC-116	ACGTAGCGTC

Out of 22 primers tested only 12 primers gave consistent RAPD profiles. The experiment was repeated twice using the primers showing amplification for confirming of the results. The rest were excluded because they were found monomorphic and sometimes the bands were found inconsistent. Amplification patterns of the polymorphic primers were pooled and a total of 420 bands with molecular weights ranging from 350 to 2000 bp were observed (Fig. 2 a, b). Highest similarity coefficient value of 86.67 % was found between isolate 2-6 and 3-4 (Table 3). The lowest similarity coefficient value (14.29%) was recorded between isolate 5-8. Cluster analysis showed a bifurcation at 25% similarity level. The isolates were grouped into 5 clusters at a similarity level of approximately 78%. Isolate 2, 3,4 and 6 were grouped into cluster, cluster B. a single representative of cluster C (isolate 5) showed approximately 45% similarity with cluster B. Isolate 1, 7 and 8 were the single representatives of cluster A,D and C

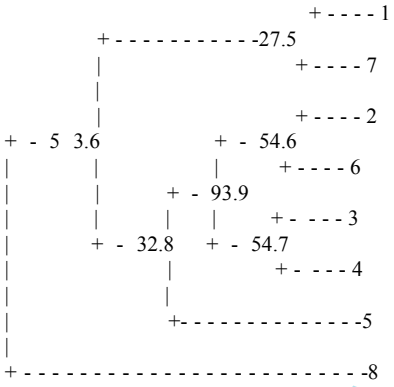

Fig. 1 : Bootstrap values of *B. bassiana* isolates as revealed by Win boot program

respectively. Bootstrapping of the values was used to determine the robustness of the data (Fig. 1). The umbers at the forks show the percentage of times that the group consisting of the species, which is to the right of that fork, occurred. Cluster 2 was found robust with a bootstrap P value of 93.9%. Regarding the original insect host of the isolates, 4 were isolated from mango mealy bug, 2 from *Sporoptera litura* and 2 from *Helicoverca armigera*. Isolates obtained from different insect host were grouped into same cluster i.e. cluster B contained isolates from all the host insects and isolates obtained from same insect were grouped into different cluster, indicating no relatedness between the host and DNA markers of the host. The pathogenicity against first, second and third instar larva of *Spilarctia obliqua* did not reveal any relatedness with the clustering pattern as different isolates representing different virulence pattern were grouped in the same cluster.

Among all the isolates, PBB-01, PBB-02, and PBB-6 isolate showed more virulence against all the age groups of *S. obliqua* followed by PBB-03, PBB-04 and PBB-08 isolates were found moderate virulence whereas PBB-05 and PBB-07 isolates were found non-pathogenic to *S. obliqua*, as less mortalities was observed in any age group of larvae.

Table 3. Simple matching coefficients of *B. bassiana* isolates as revealed by SAHN/ UPGMA tree analysis

	1	2	3	4	5	6	7	8
1	1.0000							
2	0.4375	1.0000						
3	0.3750	0.8000	1.0000					
4	0.3333	0.8125	0.8667	1.0000				
5	0.3571	0.4118	0.4375	0.4706	1.0000			
6	0.4375	0.8667	0.6875	0.8125	0.4118	1.0000		
7	0.3636	0.3333	0.3571	0.3125	0.3333	0.3333	1.0000	
8	0.2500	0.3333	0.3571	0.3125	0.1429	0.3333	0.0000	1.0000

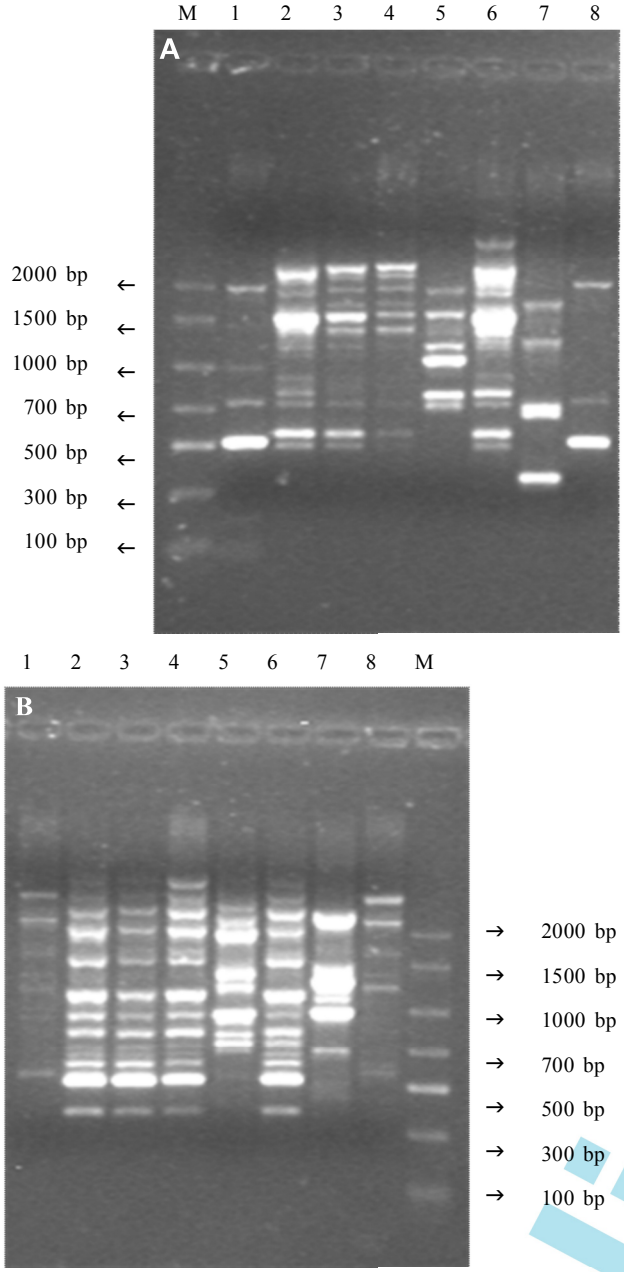

Fig. 2 : The RAPD profile of 8 *Beauveria bassiana* isolates obtained with primer (A) LC-71and (B) LC-89. Lane M is 100 bp ladder and Lanes 1 to 8 represent individual isolates from PBB-01 to PBB-08**.**

References

Anonymous: *Helicoverpa armigera* Hubner. In: Crop Protection Compendium. 2nd Edn., (CD-ROM). CAB Inter., Wallingford-Oxon (2000).

Bieliková, L., Z. Landa, L.S. Osborne, V. Curn: Characterization and identification of entomopathogenic and mycoparasitic fungi using RAPD-PCR technique. *Plant Protect. Sci.*, **38**, 1–12 (2002).

Castrillo, L.A. and W.M. Brooks: Differentiation of *Beauveria bassiana*isolates from the darkling beetle *Alphitobius diaperinus*, using isozyme and RAPD analysis. *J. Invert. Pathol.* **72**, 3, 190–196 (1998).

Castrillo, L.A., J.D. Vandenberg and S.P. Wraight: Strain specific detection of introduced *Beauveria bassiana* in agricultural fields by use of sequence–characterized amplified region markers. *J. Invert. Pathol.,* **82**, 75-83(2003).

Coates, B.S., R.L. Hellmich and L.C. Lewis: Allelic variation of a *Beauveria bassiana* (Ascomycotina: Hypocreales) minisatellite is independent of host range and geographic origin. *Genome,* **45**, 125-132 (2002).

Devi, K.U., J. Padmavathi, C.U.M. Rao, A.A.P. Khan and C.M. Murali: A study of host specificity in the entomopathogenic fungus *Beauveria bassiana* (Hypocreales, Clavicipitaceae. *Biocon. Sci. Technol.,* **18**, 975-989. (2008)

Devi, K.U., J. Padmavathi, H.C. Sharma and N. Seetharama: Laboratory evaluation of the virulence of *Beauveria bassiana* isolates to the sorghum shoot borer *Chilo partellus* Swinhoe (Lepidoptera: Pyralidae) and their characterization by RAPD – PCR. *World J. Microbiol. Biotechnol.,* **17**, 131-137. (2001).

Finney, D.J.: Probit analysis: a statistical treatment of the sigmoid response curve. 2nd Edn., Cambridge University Press, London (1964).

Gurvinder, K. and V. Padmaja: Evaluation of *Beauveria bassiana* isolates for virulence against *Spodoptera litura* (Fab.) (Lepidoptera: Noctuidae) and their characterization by RAPD-PCR. *African J. Microbiol. Res*., **2**, pp. 299-307(2008).

Hajek, A.E., S.P. Wraight and J.D. Vanderberg: Control of arthropods using pathogenic fungi. Bioexploitation of filamentous fungi. *Fungal Dive. Res. Ser.,* **6**, 309- 347 (2001).

Kranthi, K.R., D.R. Jadhav, S. Kranthi, R.R. Wanjan, S.S. Ali and D.A. Russel: Insecticide resistance in five major insect pests of cotton in India. *Crop Prot.,* **21**, 449-460 (2002).

Leal, S.C., D.J. Bertioli, T.M. Butt and J.F. Peberdy: Characterization of isolates of the entomopathgenic fungus *Metarhizium anisopliae* by RAPD–PCR. *Myco. Res.,* **98**, 1077-1081 (1994).

Lee, S.B. and J.W. Taylor: Isolation of DNA from fungal mycelia and single spores. In: PCR protocols. A guide to methods and applications. (Eds.: P.C.A. Inin, J.L. Shinsky and J.J. Wide) Academic press, San Diego, pp. 280-287 (1990).

Luz, C., M.S. Tigano, I.G. Silva, C.M. Cordeiro and S.M. Alhanabi: Selection of *Beauveria bassiana* and *Metarhizium anisopliae* isolates to control *Triatoma infestans*. *Mem. Inst. Oswaldo Cruz.,* **93**, 839- 846 (1998).

McGuire, R.M., U. Mauricio, P. Young-Hoon and H. Neal: Biological and molecular characteristics of *Beauveria bassiana* isolates from California *Lygus hesperus* (Hemiptera: Miridae) populations. *Biol Cont*. **33**, 307-314 (2005).

Rehner, S.A., E. Buckley: A *Beauveria* phylogeny inferred from nuclear ITS and EF1-a sequence: evidence for cryptic diversification and links to Cordyceps teleomorphs. Mycologia. **97**, 84-98 (2005).

Sokal, R.R. and C.D. Michener: A statistical method for evaluating systematic relationships. *Univ Kansas Sci Bull*., **38**, 1409-1438 (1958).

Valderrama, F.A.M., A.M.A. Cristancho and C.B. Cháves: Analysis of genetic variability of the entomopathogenic fungus *Beauveria bassiana* by RAPD markers. *Revista Colombiana de Entomología,* **26**, 25-29 (2000).

Valderrama F, Cristancho A, Chaver C: Analisis de la cariabilided genetica del hongo entomopatogeno. *Beauveria bassiana* con marcadores RAPD. Rev colomb entomol. **26**, 25-29 (2000).