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Characterization and Molecular Identification of the Mexican Strain CP-MABb[1] of *Beauveria* sp

Luis J Amaro Leal[1], Omar Romero-Arenas[1], Alfredo Baez Simon[1], J. António Rivera Tapia[2], Conrado Parraguirre Lezama[1], Y Primo Sanchez Morales[1*]

[1] Agroecology Center, Institute of Sciences, Benemérita Universidad Autónoma de Puebla (BUAP); Building VAL 1, Km 1,7 road to San Baltazar Tetela, San Pedro Zacachimalpa, 72960, Puebla, Mexico.

[2] Center for Research in Microbiological Sciences, ICUAP-BUAP; Building 103-J, Ciudad Universitaria. 72570, Puebla, Mexico.

ABSTRACT

Biological control is used to reduce pest insect populations and is an alternative technique to the use of synthetic insecticides. The use of entomopathogenic fungi as microbial control, has advantages over chemical insecticides, among them its high specificity on the hosts and its low environmental pollution. However, the production of biological control agents such as Beauveria bassiana, presents limitations in the characterization and identification and selection of strains with high pathogenicity, likewise the potential that native strains can have for the management of insect pests in Mexico is unknown. The CP-MABb1 obtained a 99% homology for B. bassiana of the National Center of Biotechnology Information (NCBI), and presented the highest number of conidia and a viability of 53% in PDA culture medium, representing higher production of reproductive structures in comparison to the commercial strain, virulence determinants and selection in the biological control of pests.

Keywords: Biological Control, Native Strain, Concentration of Conidia and Percentage of Viability

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Corresponding author: y Primo Sanchez Morales
E-mail ✉ primosamo@yahoo.com
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INTRODUCTION

Mitosporic fungi are cataloged as a globally recognized group of microbial agents to be used in both pest biological control programs and integrated pest management (IPM) programs. This is due to his way of acting; that it is by direct contact with the cuticle of the host and by the availability of technologies to massively produce them [1].

It has been demonstrated that in order to obtain a highly effective product to control a pest species, it must be based on a wide range of isolates, selected in principle in terms of their virulence [2]. Isolates that cause spectacular epizootics, as is the case of some of the imperfect entomopathogenic fungal genera, are promising candidates for use in pest control, as long as they achieve narrow host specificity, good tolerance to adverse environmental factors, ease of production, behavior in adequate storage and high safety in mammals [1].

Entomopathogenic fungi are a group of microorganisms with more than 700 species within 90 genera that can infect insects [3]. Diseases caused by fungi in insects commonly reduce populations, demonstrating that bioinsecticides can be a viable alternative to solve the problems of insect pests in agriculture [4]. Among the fungi most commonly used as biological insecticides are *Beauveria bassiana* and *Metarhizium anisopliae*, where B. bassiana infects more than 200 species of insects and mites of various genera, in the order Orthoptera, Hemiptera, Lepidoptera, Dermaptera, Hymenoptera, Coleoptera, others [5, 6].

For the use of *B. bassiana*, as a biological control agent, it is necessary to develop mass production and formulation systems that allow greater

or equal efficacy than chemical products, being one of the requirements for the acceptance and commercialization of a biological product, in addition develop formulations that guarantee their viability, development capacity and storage [7]. Therefore, in the present study, one native isolation of Puebla, Mexico was characterized in order to select the best physiological and pathogenic characteristics for biological control.

MATERIALS AND METHODS

Biological material

The strain used in this research is CP-MABb1, which comes from the Municipality of Tetela de Ocampo, Puebla-Mexico; it conserved in dextrose agar culture medium (PDA) in the Center for Genetic Resources of the Agroecology Center of the Institute of Sciences of the BUAP. The identity of the strain was confirmed by the amplification of the ITS-1 and ITS-4 region. In the program MEGA 7.0, was constructed phylogeny with the Neighbored Joining algorithm with bootstrap of 1000 replicas, considering the model of nucleotide substitution of Kimura 2-p and Maximum Paternity to infer phylogenies through the method of union of neighbors [8, 9].

The study included a strain commercially of the *B. bassiana* available in the city of Puebla-Mexico from the company "Agrobionsa de Mexico", where the B-BLAS product was purchased at a concentration of $1x10^8$ conidia/mL and a viability 94%. Subsequently, it was reactivated in medium Sabouraud-Dextrose-Agar (SDA) plus 0.5 mg l^{-1} of Chloramphenicol at 27±1 °C in Petri dishes of 9 cm in diameter.

Identification of *B. bassiana*

Genomic DNA extraction. From plates with PDA with the monosporic cultures of 72 hours of incubation, scraping was performed for their extraction of DNA with the ZR Fungal/Bacterial DNA MiniPrep kit (ZIMO Research D6005). The DNA was run on a 1% agarose gel, in a horizontal electrophoresis chamber (BIO-RAD) at 70 volts for 60 minutes using an EC105 power source. The extractions of genomic DNA were conserved at -20 °C.

PCR amplification of the conserved ITS1 and ITS4 regions of strain CP-MABb1

PCR reactions were performed in a final volume of 50 µl, using 1 µl of genomic DNA, 5 µl of 10x Buffer, 1.5 µl of 50 mM magnesium chloride (final 1.5 mM), 1 µl of 10 mM dNTPs (0.2 mM), 1 µl of each of the primers; the oligonucleotides used were the ITS-1 region (5'TCCGTAGGTGAACCTGCGG3 ') and ITS-4 (5'TCCTCCGCTTATTGATATGC3') [8] plus 5 µM (final 0.1 µM) and 0.4 µL of the enzyme Taq DNA polymerase 5 U/µl and completing the volume at 50 µl with sterile milliQ water. Subsequently, the amplification was performed with 1 cycle of 5 min at 95°C, 37 cycles of 30 secs at 95°C, the hybridization temperature varied for each primer; 50°C (AG and AG), 55°C (CCA, TG and CT) and 58°C (GT) for 45 seconds. After a cycle at 72°C for 2 min, a final extension step of 7 min was finally given at 72ºC, keeping at 4ºC. Once the products were obtained, they were purified using the Wizard® DNA Purification Kit.

Sequencing

The amplified product of CP-MABb1 was poured into a 0.2 ml Eppendorf tube with approximately 20 µl of final product, at a concentration of 50 ng. The sample was sent for sequencing in both directions to the Biomolecular Detection Center of the Benemérita Autonomous University of Puebla (BUAP). The sequence obtained for each genomic region of the CP-MABb1 was compared in silico in its homology with those existing in the public BLAST database, present in the Web site of the National Center of Biotechnology Information (NCBI) of the United States http://www.ncbi.nlm.nih.gov/BLAST [10].

Growth rate and Development rate

The growth rate and development rate of the *B. bassiana* strains were determined in Petri dishes (4.5 cm in diameter) in culture medium (PDA), incubated at room temperature for 10 days, the speed of growth it was measured every 24 hrs and the macroscopic morphological characteristics of the colonies were recorded in terms of texture, density, aerial mycelium and color. The rate of growth and the rate of development were determined using the following formula: TD = VCF-VCI / No. of days.

Preparation of bio-preparations and viability of CP-MABb1

Preparations of CP-MABb1 from *B. bassiana* were taken in PDA cultivation medium of 10 days of development with a photoperiod of 12:12 light-dark hours, then massively reproduced in 300 g of rice sterilized in polipal bags; for this, 5 Petri dishes previously colonized, each

box was placed 5 mL of sterile water and with the help of a bacteriological handle was mixed with conidia. Then, inoculated 5 mL of suspension of conidia to rice sterilized under aseptic conditions in the isolation chamber and incubated for 20 days at 28±1 °C. To obtain the spores, 500 ml of sterile distilled water was added, gently rubbing the rice. The concentration of conidia in a 1/10 ratio was determined by counting in a Neubauer chamber, adjusting the initial suspension with $1x10^8$ conidia/ml, the obtained suspension was kept in a refrigerator until its analysis [11]. Subsequently, 3 concentrations were prepared (Table 2), using the suspension obtained from conidia of CP-MABb1 and B-BLAS from *B. bassiana*, to determine the best viability of conidia, at different concentrations. For the evaluation of the viability of conidia, the methodology of Marin and Bustillo (2002) was followed, where five points were placed with a suspension of $1x10^8$ conidia/ml in Petri dishes (100 x 15 mm) with PDA. 100 conidia were observed and the number of germinated conidia of the five points was recorded and this value directly represented the percentage germination rate of an experimental unit.

RESULTS AND DISCUSSION

Identification

The morphological identification of the CP-MABb1 strain was carried out with the purpose of corroborating the taxonomic classification of strain CP-Bb1, which presents smooth and cenocitic hyphae, with conidiogenic cells forming dense clusters irregularly grouped; the fialides are swollen in the base that resembles the structure of a sub-globose flask and become thinner towards the part of the rachis. The size of the conidiogenic cells is 3.6 x 1.5 µm, the spine is 2.2 x 1 µm. The conidia are hyaline globose with a size of 2.3 µm in diameter, these results coincided with the description of Alean [12] and Domsch et al., [13] for the species *B. bassiana*.

Amplification and Sequencing PCR-ITS

With the amplification of the ITS-1 and ITS-4 region, a product of 521 bp was obtained (Fig 1).

The sequence of these fragments was compared with the GenBank data, where the CP-MABb1 presented a high degree of identity as *B. bassiana.* A low level of sequence variation was detected within the ITS region, which corroborates other authors in the present study [14-17].

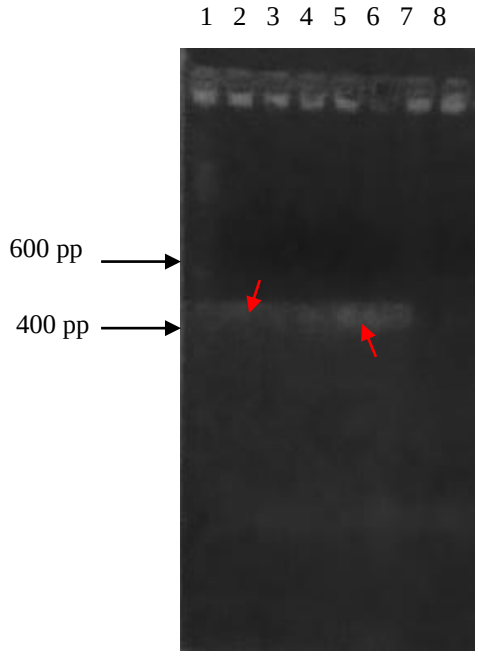

Fig. 1. Lane 1: molecular weight marker 1 kb (Promega, USA), Lane 2 to 4: amplification of the internal spacer of the transcript (ITS) of the ribosomal DNA, with the combination of the primers ITS1 and ITS4 (size approximate of the band: 520 to 610 bp) of the CP-MABb1 strain in triplicate, Lane 5 to 7: amplification of the internal spacer of the transcript (ITS) of the ribosomal DNA, with the combination of the primers ITS1 and ITS4 of the commercial strain B -BLAS in triplicate, Lane 8: negative control.

The sequenced PCR product was compared to the fungal sequences published through the BLAST nucleotide-nucleotide homology search program. This one showed an identity with the 521 nucleotides of 99% with *B. bassiana*. This sequence was included in the database of the National Center for Biological Information (NCBI) with the access number 06YSRHAB015.

Phylogenetic trees (Figs 2 and 3) were constructed with 9 accessions of *B. bassiana* available in the database (NCBI) of the 18S ribosomal region of *B. bassiana*.

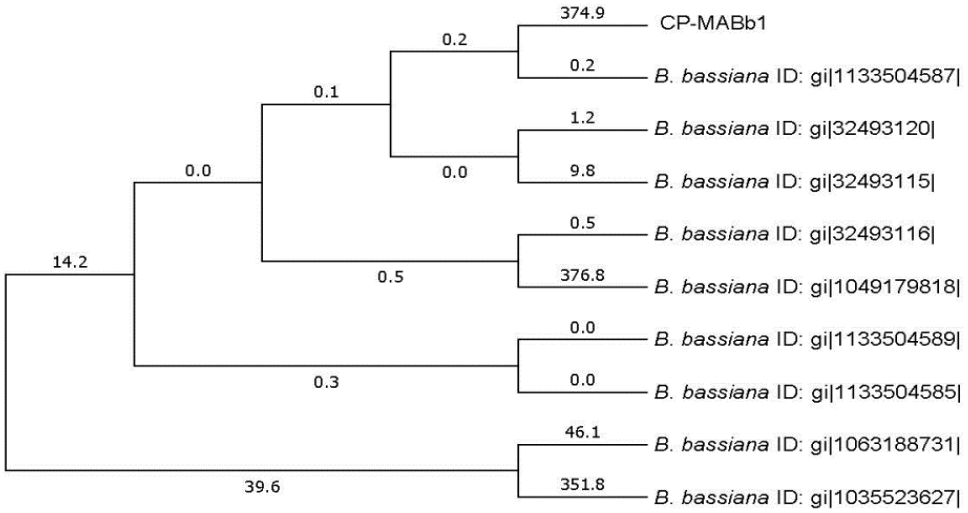

Fig. 2. Phylogenetic tree of the CP-MABb1 generated by the Maximum Paternity method, with a length of 1216. It has a consistency index of 0.753289 (0.603699%), a retention index of 0.096386 (0.096386%) and a composite index of 0.072606 (0.058188%). The analysis involved 9 nucleotide sequences from that of the (NCBI) database of the 18S ribosomal region of *B. bassiana*.

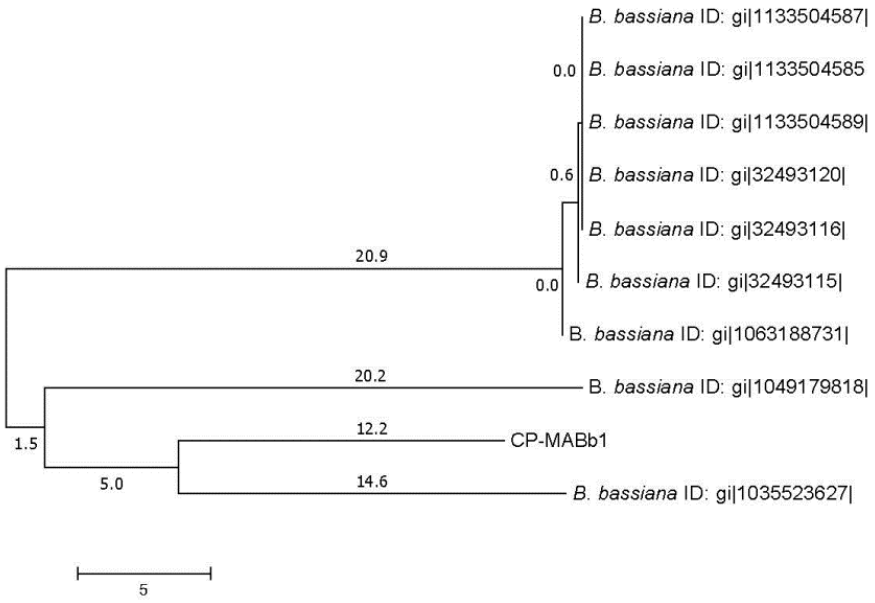

Fig. 3. Phylogenetic tree of CP-MABb1 generated by the Neighbor-Joining method, with the sum of the length of the branch of 75.07335039. Evolutionary distances were calculated using the maximum likelihood method composed of 9 nucleotide sequences from the database (NCBI) of the 18S ribosomal region of *B. bassiana.*

Growth rate and Development rate

Both strains were characterized by a slow radial growth, reaching 23-25 mm in diameter in 10 days. The CP-MABb1 presented woolly mycelium, aerial and abundant, as well as a white color at the beginning of its development, turning yellow after 10 days in PDA medium, unlike the commercial strain. Significant differences were registered in the t-Student test (t=18, P=0.074) in the mycelial growth rate (RMG) between the

CP-MABb1 and the commercial strain (Table 1). The highest RMG was the CP-MABb1 strain with 3.28 cm, while the B-BLAS strain showed the lowest RMG with 2.92 cm, which coincides with that established by Domsch et al., [13] for *B. bassiana*, which has a range of 0.6 to 2.3 cm in diameter.

Table 1. Macroscopic characteristics of *B. Bassiana* strains in PDA medium

Culture Medium	Key	Texture	Density	Aerial Mycelium	Color	Radial Growth (cm/day)	Developmet Rate (mm/day)
PDA	CP-MABb1	Woolly	Abundant	Yes	White / yellow	3.28 ±0.03*	4.8 ±0.05*
	B-BLAS	Velvety	Regular	Not	White	2.92±0.1	2.6±0.33

* The averages indicate significant differences with the t-Student test (P <0.05).

Preparation of suspensions and viability of conidia

A significant effect of the treatments was observed on the concentration of conidia (ANOVA of one factor, F=3.1, P=0.067). Conidia production of *B. bassiana* has been developed in several countries with a high level of production efficiency [18].

The highest concentration of conidia was obtained in the CP-MABb1 with the concentration [1/1] with 3.67x10^8 conidia/ml with a viability of 53% (Table 2). The lowest concentrations were obtained in the commercial strain of B-Blas with the concentration [1/10] with 0.70x10^8 conidia/ml, with a viability of 11%. Feng et al., [19] reported 7-8 x 10^{12} conidia/kg of rice, similar results to the present investigation. By selecting biological preparations with rapid germination and high viability of conidia increases the probability of success in the field; In contrast, isolates with slow germination and low viability are more sensitive to environmental variations. The speed and viability of germination are the most reported virulence determinants [20].

Table 2. Concentration and viability of conidia of strain CP-Bb1 of *B. bassiana*

Key	Concentration	Conidia x10^8 / ml*			% Viability *		
CP-MABb1	1/1	3.67	±	0.15 a	53.00	±	0.57 a
	1/5	3.37	±	0.12 b	47.00	±	0.57 a
	1/10	2.78	±	0.12 b	34.00	±	0.57 b
B+ Blas (Control)	1/1	2.57	±	0.20 c	35.00	±	0.57 b
	1/5	1.70	±	0.06 d	23.67	±	0.33 c
	1/10	0.70	±	0.06 e	11.00	±	0.57 d

* Averages with different letters in the column indicate significant differences with the Tukey-Kramer test (P<0.05).

CONCLUSIONS

An extension of 521 base pairs of the conserved regions ITS1 and ITS4 of the strain CP-MABb1 was obtained, whose sequence allowed to identify the homology of 99% for *Beauveria bassiana*. The highest production of conidia was presented in the native Mexican strain with 3.67x108 with/ml and a viability of 53% in PDA culture medium, representing higher production of reproductive structures compared to the commercial strain.

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