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Cytotoxic Activity of Fungal Metabolites from the Pathogenic Fungus *Beauveria bassiana*: An Intraspecific Evaluation of Beauvericin Production

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Abstract The cyclohexadepsipeptide beauvericin (BEA) is a mycotoxin produced by the fungus *Beauveria bassiana* (Bals.). Using ELISA, different accessions of *B. bassiana*, belonging to distinct genetic groups, were analyzed to determine their variability in BEA production. The cytotoxic effect of pure mycotoxins and crude extracts was also tested on insect cell lines SF-9 and SF-21. The results showed that BEA production was significantly different between all strains. Bb 9024 exhibited the highest levels (98.56 mg/l), while Bb 9001 the lowest (15.66 mg/l). Statistical difference was found when BEA CC_{50} values (2.81 and 6.94 μM) were compared with those values from others mycotoxins (4.23–11.95 μM). Although no correlation has been observed between beauvericin production and phylogenetic grouping, the results suggest a comprehensible involvement of these metabolites during the infection process. The biological evaluation of metabolites produce by entomopathogenic fungi provides better criteria to design more effective formulations for pest management.

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Introduction

The genetic diversity of entomopathogenic populations represents an alternative in order to explore valuable microorganisms with better efficiency of insect control at field conditions [24]. A former molecular characterization of variability in 95 *B. bassiana* strains indicated a low but significant degree of genetic diversity, which is not linked to geographic distribution [8]. In addition to some virulence tests, genes and metabolites of interest have been also screened to design new formulations that combine multiple mechanisms used by the fungus during the infection process. These mechanisms include the production of secondary metabolites such as the mycotoxins BEA, tenellin, bassianin, beauveriolide, oosporein and the recently described bassiacridin [18]. Mycotoxins are relatively small molecules that play an important role in fungal infection and can generate a toxic response in different organisms [22]. The ability of *B. bassiana* to overcome host defenses and eliminate bacterial competition has been demonstrated to be due in great part to toxin production [19].

The mycotoxin beauvericin was first discovered in *B. bassiana* [12]. It is a cyclohexadepsipeptide, consisting of three *N*-methyl phenylalanine molecules alternated with three hydroxyisovalerate acid molecules. The mycotoxin can be produced by other species of the genus *Beauveria*, such as *Beauveria brongniartii,* and by other genera [9]. Although these entomopathogens may display similar biological actions on the host insects, studies indicate that the complex profile of mycotoxin production in *Beauveria*

prevents the prediction of pathogenicity and virulence [17]. Variations in mycotoxin production by entomopathogenic fungi have been previously studied [15]. A large interspecific and intraspecific variation was reported in the production of destruxin by *Metarhizium anisopliae*, an important toxin that gives the fungus biological efficacy as insecticide [1]. In this work, we have measured not only the variability in BEA production among the most important accessions belonging to discrete genetic groups of *B. bassiana* but also the cytotoxic effect of other well-known fungal metabolites on insect cell lines.

Materials and Methods

Beauveria bassiana Strains

Beauveria bassiana Bb 9001, Bb 9005, Bb 9010, Bb 9011, Bb 9016, Bb 9023, Bb 9024, Bb 9119, Bb 9205; one strain of *B. brongniartii* (Bbr 9301); and one of *M. anisopliae* (Ma 9236) were obtained from the microorganism collection at the National Coffee Research Center, CENICAFE, Colombia. All strains had been previously characterized using RAPDs and ITS-RFLPs molecular markets and represent the main phylogenetic groups reported by Gaitán et al. [8]. Reactivation from 0.1 ml of stock media (10% glycerol at −70°C) was made by plating on Petri dishes with Sabouraud dextrose agar (SDA) media supplemented with 0.4% cuticle of coffee berry borer.

Preparation of Crude Extracts

Flasks with 100 ml of Sabouraud liquid medium at pH 6.8, containing 0.1% chitin, were adjusted to a concentration of 1.4×10^7 viable spores/ml collected from dishes with all strains including Ma 9236; the flasks were incubated in an orbital shaker (150 rpm), at 27 ± 1°C, 80% relative humidity and darkness for 9 days. Liquid cultures were filtered through Whatman #1 paper using vacuum in order to separate the crude extract from the mycelium and spore mass. Total soluble protein from liquid cultures for each strain was quantified using Branford's protocol [2] using a UNICAM UV/VIS 2 spectrophotometer at 595 nm. Finally, filtered liquid cultures were centrifuged at 12.000×*g* for 30 min, and the supernatant was then used for serological tests.

ELISA Tests

Microtiter plates were coated with 200 µl of liquid supernatant from *B. bassiana* cell cultures for 24 h at 8°C and blocked in 5% skim milk for 2 h and washed with TTBS (50 mM Tris–HCl, pH 8.0, 138 mM NaCl, 0.05% Tween 20) for 30 min. The plate was incubated with a rabbit polyclonal antibody serum raised against BEA as primary antibody solution 1:1000 for 2 h at 37°C and washed in TTBS. A goat anti-rabbit IgG immunoglobulin G coupled to horseradish peroxidase (Bio-Rad) was used as the secondary antibody 1:2000 at the same conditions. Finally, a 0.01% (w/v) solution of 3,3′,5,5′-tetramethylbenzidine in TBS containing 5 µl H_2O_2 was used as substrate. The absorbance was determined at 490 nm. BEA concentration (mg/l) was calculated through a correlation between the sample absorbance and the curve of known concentrations constructed following the methodology above described [ABS = 0.235 (BEA concentration) + 0.0268]. Each assay was carried out with five replicates.

Mycotoxin and Reagents

The following standard fungal metabolites: oosporein (OOS), tenellin (TEN) and bassianin (BAS) with 90% of purity was a kind gift from Dr. Leo C. Vining (University of Dalhousie, Canada). BEA with 97%, the reagents namely TNM-FH, heat-inactivated fetal calf serum (FCS), antibiotic solution, dimethyl sulfoxide (DMSO) and trypan blue dye solution 0.4% were purchased from Sigma (St. Louis, USA).

Insect Cell Cultures and Treatments

Lepidoptera (*Spodoptera frugiperda*) cell lines SF-9 and SF-21, obtained from the envelope of pupal ovaries and primary explants from pupal tissues, respectively [23], were maintained at 27°C in TNM-FH medium supplemented with 10% FCS and 1% penicillin–streptomycin–amphotericin solution. Cultures in the early stationary phase (typical cell density with cell viability approximately 85%) were split every 5 days with a starting density of 2×10^5 cells/ml. The fungal metabolite standards were dissolved in methanol with the exception of BAS and TEN that were dissolved in DMSO. The standard solutions were tested at concentrations ranging from 1 to 100 µM. Supernatants obtained from liquid cultures of Bb 9024, Bb 9205 and Bb 9001 strains selected by BEA production were tested at concentrations ranging 1–100 ppm. Appropriate controls were prepared in the same manner without toxins (liquid media) and solvents with final concentration of 1%. Afterward, the insect cells were seeded in 12-well cell culture plates at the early stationary phase (1×10^6 cells/ml), and test solutions were added at a ratio of 1:100 (v/v). Plates were finally incubated for 48 and 72 h at 27°C.

Bioassays: Cell Viability

Trypan blue dye (0.4%) was added to insect cell cultures in a ratio of 1:1 and preparations were viewed at 100× using standard light microscopy. The ratio of live to dead cells

(dyed) was resolved with standard curves and 50% cytotoxic concentration (CC_{50}), which is the concentration of mycotoxin that caused a 50% decrease in cell viability expressed in μM. Additionally, the determination of lethal concentration that caused 50% mortality values (LC_{50}) for the crude extracts assays was also carried out and expressed in ppm. Three independent assessments with five replicates to all bioassays were evaluated.

Statistical

The experimental unit consisted of a flask containing the corresponding strain. In all cases, five replicates per strain (treatments) were analyzed. For statistical analysis, one-way variance analysis (ANOVA) and Duncan comparison test between treatments and controls were performed. Insect cell viability statistical analyses were performed with SigmaStat 2.03 for Windows (SPSS Inc., USA), and nonparametric Tukey comparison tests were used to compare statistical differences for values obtained for CC_{50} and LC_{50} considering differences at $P \leq 0.05$ and $P \leq 0.005$, respectively. LC_{50} values and their lower and upper confidence limits (confidence 95%) were calculated with the probit statistical program.

Results

BEA Production Variability

The highest concentration of BEA was observed in Bb 9024 and Bb 9010 with 98.60 and 98.30 mg/l, respectively. On the contrary, Bb 9119 and Bb 9001 came out as the lowest producers with 18.81 and 15.66 mg/l, respectively (Table 1). All strains had significant statistical differences when compared with the control (Ma 9236) and also between them, allowing the identification of three well-differentiated groups (Table 2). Thus, the highest producers of this toxin were Bb 9024 and Bb 9010. The strains Bb 9011, Bb 9205, Bb 9005, Bb 9023 and *B. brongniartii* 9301 showed a medium level and Bb 9016, Bb 9119 and Bb 9001, the lower expression level.

Table 2 Classification by groups from levels of toxin production (Duncan test 5%)

Duncan test	Average	N	Group
a*	98.43	10	I
b	44.12	25	II
c	20.79	15	III

N total number of values by data set (Group)

* Means with the same letter are not significantly different from each group

However, the Bb 9205 strain produced the highest levels of total protein production, while the Bb 9023 strain produced the lowest level, both belonging to the group II (middle toxin production). In addition, it was found that the strains Bb 9024 and Bb 9010, belonging to the first group I of toxin production showed the lowest levels of protein production when compared with the group II of toxin production (183.72 and 133.19 mg/l, respectively) (Table 1). In relation to the BEA production per gram of soluble total protein, Bb 9010 strain showed the highest values while Bb 9205 strain was located into group of the least production relating these variables, both proceeding from different genetic group. In addition, Bb 9023 and Bb 9024 strains belonging to the same genetic group showed very similar levels of specific toxin production (Fig. 1).

Cytotoxic Effects of the Pure Mycotoxin on Insect Cell Viability

All *B. bassiana* metabolites selected for this study (BEA, OOS, BAS and TEN) showed effects on the lepidopteran cell lines SF-9 and SF-21. Metabolites that differed from BEA showed CC_{50} values ranging between 4.23 and

Table 1 BEA production in strains tested

Strains	Origin	BEA production (mg/l)	Total soluble protein (mg/l)
Bb 9001	Colombia (Nariño)	15.66 ± 3.57^{c}	355.73 ± 10.89^{e}
Bb 9005	Colombia (Nariño)	37.53 ± 13.25^{b}	204.54 ± 33.85^{cd}
Bb 9010	Colombia	98.30 ± 8.79^{a}	133.19 ± 24.61^{b}
Bb 9011	Colombia (Huila)	69.62 ± 20.86^{b}	198.76 ± 24.86^{cd}
Bb 9016	Thailand	27.92 ± 4.74^{c}	166.21 ± 31.21^{bcd}
Bb 9023	Philippines	35.23 ± 2.67^{b}	56.69 ± 35.12^{a}
Bb 9024	Canada	98.56 ± 19.83^{a}	183.72 ± 45.39^{cd}
Bb 9119	Colombia (Risaralda)	18.81 ± 6.39^{c}	156.59 ± 28.26^{bc}
Bb 9205	Colombia	45.79 ± 21.29^{b}	475.40 ± 67.39^{f}
Bbr 9301	Colombia (Santander)	32.43 ± 4.20^{b}	213.53 ± 36.60^{d}
Ma 9236	Colombia	0.00	201.38 ± 23.55^{cd}

Means with the same letter are not significantly different (Duncan 5%). Data are expressed as mean ± standard deviation

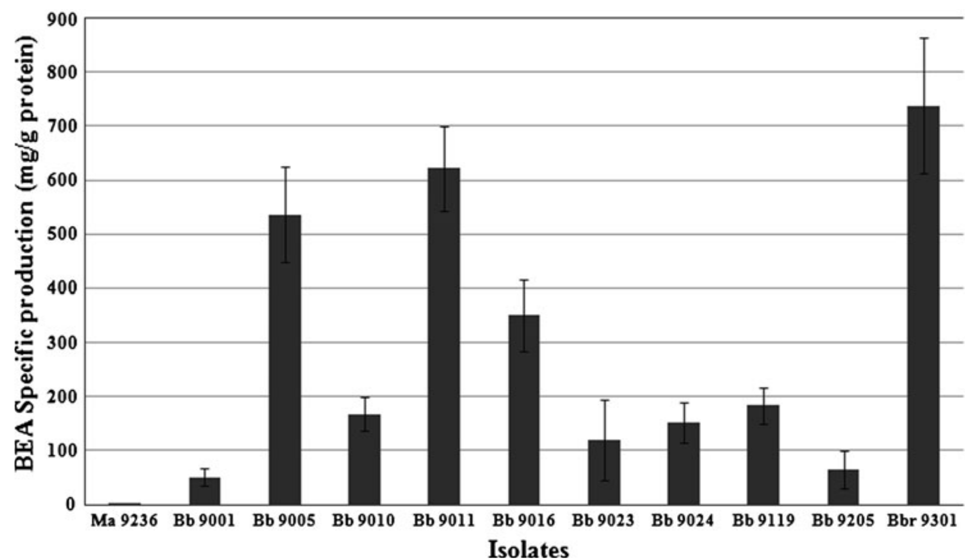

Fig. 1 BEA concentration in *B. bassiana* culture extracts

4.84 μM with SF-9 and 10.43 and 11.95 μM with SF-21 (Table 3). After 48 h of exposure, BEA exhibited statistical differences in toxic activity on both insect cell lines in comparison with the other metabolites that were tested with CC_{50} of 2.81 and 6.94 μM for SF-9 and SF-21, respectively. Likewise, the cell viability of both insect cell lines that was obtained from dose–response curves decreased as the toxin concentration increased, with a statistical difference at 10 μM ($P \leq 0.05$; Fig. 2). It is important to point out that a lower cytotoxic effect on SF-21 insect cell lines was observed in all assessments.

Effects of the Crude Extracts from Different *B. bassiana* Strains and Their Mixture on Insect Cell Viability

The *B. bassiana* strains selected for these assays belonging to different groups of BEA production were previously characterized. The results showing the effects on cell line SF-9 at 48 and 72 h for all crude extracts are summarized in Table 4. After 48 h of exposure, LC_{50} values for all treatments were above 100 ppm ranging 112–316 ppm. However, after 72 h of exposure, all treatments increased the effect over cell line with LC_{50} values ranging 31–145 ppm, except Bb 9205 with a LC_{50} of 451 ppm. Likewise, Bb 9024 and Bb 9001 showed stronger effects on cell line SF-9 with LC_{50} values of 31 and 37 ppm, respectively, with no statistical differences. However, statistical differences for Bb 9205, the mixture assay and the other strains were observed at 48 h ($P \leq 0.005$). In addition, the susceptibility of cell line SF-9 to crude extract obtained from *B. bassiana* fungus was demonstrated. The results suggested that there was a high variation not only in BEA production but also in the content of other metabolites that facilitate the cytotoxic activity.

Discussion

Our results showed a high variation in BEA production among the different strains that were selected. Similar results were obtained by Gupta et al. [10] and Moretti et al. [15] working with *B. bassiana* and *Fusarium* spp. Furthermore, recent studies evaluating BEA and enniatins (K and I) from *Fusarium* species also have demonstrated a high level of diversity [21]. A survey of 24 strains in *B. bassiana*, made by Peczynska et al. [16], resulted in a very low frequency (12.5%) of strains producing BEA. The present study confirms the intraspecific variability in the production of this toxic peptide (Table 1), but no correlation has been observed between beauvericin production and phylogenetic grouping. This study allowed us to determine the production of the mycotoxin, as a key aspect of variability in *B. bassiana*, and to evaluate its participation at the infection. BEA represents the main toxin that is produced by *Beauveria* spp. [10]. For this reason, the cytotoxic effect of the pure fungal metabolites (BEA, OOS, BAS and TEN) and crude extracts batches from *B. bassiana* fungus on lepidopteran cell lines SF-9 and SF-21 were tested. A direct dose–response correlation between cell viability and toxin concentration was registered when the insect cell cultures SF-9 and SF-21 were exposed to BEA. In addition, statistical difference was found between 1 and 5 μM when compared with the high toxin concentration that was used (10 μM; $P \leq 0.05$; Fig. 2). The effect of the toxin treatment obtained with SF-9 was comparable with those obtained with *A. salina* [13], *S. frugiperda* [7] and other registered with fungal metabolites from *Fusarium* sp. [11].

Previous studies with BEA were carried out on *murine* sp. and human cellular lines showing high toxicity on the target cells [4] and on *S. frugiperda* cell lines [7]. To date,

Table 3 Cytotoxicity induced by fungal metabolites on SF-9 and SF-21 insect cells, using trypan blue dye exclusion test after 48 h

Fungal metabolites	CC_{50} (μM) SF-9	CC_{50} (μM) SF-21
Beauvericin	2.81 ± 0.37*	6.94 ± 0.91*
Oosporein	4.23 ± 0.46	10.43 ± 1.14
Bassianin	4.91 ± 0.50	12.12 ± 1.24
Tenellin	4.84 ± 0.31	11.95 ± 0.76

Data are expressed as mean ± standard deviation from three independent assessments with five replicates each

* Value differs significantly ($P \leq 0.05$)

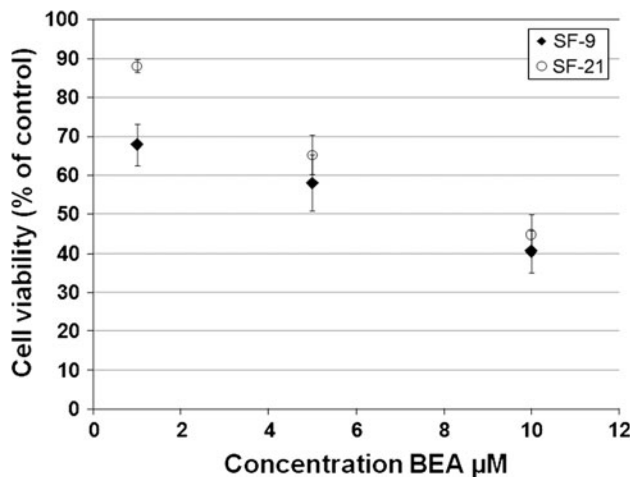

Fig. 2 Dose–response obtained with BEA after 48 h of exposure; bars represent standard deviation of the mean cell viability (five replicates). To each cell line, significant differences between concentrations are indicated by different letters Tukey ($P \leq 0.05$)

Table 4 Toxicity of different crude extracts from *B. bassiana* and its combination on SF-9 insect cell using trypan blue dye exclusion. (LC50 values with lower and upper fiducial limits (95%) as calculated by probit. (SPSS Inc., US)

Crude extract	LC_{50} (ppm)	
	48 h incubation	72 h incubation
Bb 9205	264 (79; 633)[bc]	451 (153; 1,570)[c]
Bb 9024	190 (n.c.)[b]	31 (n.c.)[a]
Bb 9001	112 (73; 233)[a]	37 (n.c.)[a]
Mix (Bb 9205 + 9024 + 9001)	316 (n.c.)[c]	145 (87; 384)[b]

n.c. Fiducial limits no reliable

* To each evaluation, significant differences between concentrations are indicated by different letters Tukey ($P \leq 0.005$)

this is the first report showing the effect of the pure toxins (OOS, BAS and TEN) and crude extracts produced by *B. bassiana* fungus on lepidopteran cell lines. Based on the results from insect cell viability assays, it was found that the CC_{50} value produced by those toxins was approximately two times more than the CC_{50} value calculated for BEA to both insect cell lines SF-9 and SF-21(Table 3). Similar CC_{50} values (3.0 and 2.5 μM) were reported by Fornelli et al. [7] and by Calò et al. [3] working with BEA toxin on SF-9 insect cell line. Our results also show that the cell line SF-9 is weakly more sensitive than SF-21. Furthermore, it has been demonstrated that BEA has not only antimicrobial and inhibitory effects but also insecticidal properties that generate differential insect cell susceptibility when treated with this toxin [14].

A direct relation between the toxin amount and its pathogenicity was previously reported [27], causing a progressive degeneration of the host tissues and structural changes of the membranes and also generating changes in the electrical activity of the nervous system. This modification was caused by an increased consume of oxygen as an insect attempt to get recover from the toxic effect [6]. Zizca and Wiser [28] corroborated the BEA effect on *Culex pipiens* larvae. They found that low toxin concentrations (0.1 μg/μl) applied topically on insects cause 44% of mortality in just 48 h. In addition, some authors have also reported in vivo effects of fungal metabolites as agents for control of insects [25]. Some of those results show that the main symptom from insect intoxication was the generalized vacuolization and the toxic effect on the mitochondria, which swelled and took aspect of spherical vacuoles. The same effect on insect cell lines was observed in our assays under 100× light microscopy. Moreover, it was observed generalized stress and cellular lyses. However, no evidence of DNA disruption by the apoptosis process to all treatments was observed after electrophoreses (data not shown). Commonly, in this process, the nucleus chromatin is concentrated in granules localized throughout the nuclear membrane. Results obtained by Gupta et al. [9], applying BEA on *Leptinotarsa decemlineata* larvae, found mortalities ranging between 50 and 90% at 48 h at concentrations of 633 and 1,196 ppm.

In spite of the fact that the insecticidal action of the toxin has been broadly demonstrated, further research is needed to evaluate its real potential for pest control. The secondary metabolites produced by *B. bassiana* have a great potential as a natural source of different kind of substances to control pest and diseases or as new drugs [14]. Our results suggest a clear involvement of metabolites from *B. bassiana* fungus at the final infection process through its toxic activity on insect cell viability (Table 4). It is also possible that synergy and antagonism among fungal compounds could also influence the overall toxicity of the crude extracts [20]. Although the variability in BEA production has been demonstrated, strains representing each production group have been selected. No correlation

on cell viability of the crude extract batches from *B. bassiana* and toxin production was observed. Xu et al. [26] revealed that BEA plays a highly significant but not indispensable role in virulence using knockout *B. bassiana* strain in infection assays. For example, Bb 9001 exhibited lower BEA production (15.66 mg/l) but a stronger effect on insect cell viability (SF-9) with an LC_{50} value of 112 ppm at 48 h and 31 ppm at 72 h of exposure. In a previous study, destruxin A (DTXA) from *M. anisopliae* showed the most toxic effect on SF-9 insect cells, with a LC_{50} value of 5 ppm. However, 500 ppm of the crude extracts containing 90 ppm of DTXA was less effective and did not cause such a high mortality [20]. Crude extract from Bb 9205 at 72 h and the mixture (Bb 9205 + 9024 + 9001) at 48 h resulted in less cytotoxicity ($P \leq 0.005$) when compared with the other crude extracts that were tested. Though, treatments with Bb 9024 and Bb 9001 decreased the insect cell viability at 72 h.

In general, the biological action of BEA is related to its ionophore property, by complexing with alkaline metals BEA creates "holes" in the cellular membrane and affects the capacity of the cell to transport and exchange substances with its environment. All these aspects must be considered in further works in order to implement and formulate biological products that involve more than one strain from a specific microorganism [5]. On the basis of our results, a complete characterization of *B. bassiana* for mycotoxin production and cytotoxic effect of the fungal metabolites on insect cell lines will allow to get a better selection of fungal strains, which should be more efficient for the biological control of insect pests.

Conclusions

The cytotoxic effect of BEA and pure fungal metabolites (OOS, BAS and TEN) was demonstrated. The genetic variability for the *B. bassiana* strains regarding BEA production was also corroborated. Our findings suggest that the cytotoxic activity of crude extracts and mycotoxin from different strains of *B. bassiana* cannot be attributed to just one toxic compound alone. Instead, the toxic effect could be the action of multiples metabolites including those mycotoxins that were tested in our experiments. The results of this study constitute a valuable aid in searching for biomolecules with high potential to be used in agriculture in order to implement appropriate solutions for pest control through biotechnological tools that allow us to prepare better formulations.

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