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Enzymatic and antagonistic potential of bacteria isolated from typical fruit of Cerrado in Minas Gerais State, Brazil

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ABSTRACT. Brazil has great biodiversity, which is observed in the Cerrado biome of the tropical Brazilian savanna. The objectives of this study were to isolate and identify bacteria from *Psychotria hoffmannseggiana* with potential cellulase and pectinase production and with antagonistic activity against *Aspergillus carbonarius*, an Ochratoxin A (OTA) producer. Ripe fruit were collected in the region of Passos City in the preserved Cerrado area in Minas Gerais State, Brazil. Serial dilutions were performed, and the bacteria isolated were biochemically characterized and identified by sequencing. To analyze the production of enzymes, the bacteria were cultivated in CMC and pectinase media. The better enzyme producers were optimized for production. Assays on the antagonistic activity for growth and sporulation were carried out in co-culture (bacteria and filamentous fungi). TLC was performed to verify the mycotoxin production. The predominant microbiota were Gram-negative bacteria belonging to the *Enterobacteriaceae* family. Some isolates showed potential for enzymatic and antagonistic activity, especially the isolate identified as *Lysinibacillus fusiformis*. This species was a better producer of cellulases (maximum activity: 103.1 mg glucose $min.^{-1}$ mg^{-1} protein). In conclusion, the bacteria isolated from *Psychotria hoffmannseggiana* showed biotechnological potential for agro-industry and the environmental aspect.

Keywords: microorganism, *Psychotria hoffmannseggiana*, biotechnological application.

Potencial enzimático e antagonista de bactérias isoladas de fruto típico do Cerrado em Minas Gerais, Brasil

RESUMO. O Brasil tem uma grande biodiversidade no bioma Cerrado típico de savanas tropicais. Os objetivos deste estudo foram isolar e identificar bactérias celulolíticas e pectinolíticas e com atividade antagonista contra contra *Aspergillus. carbonarius* produtores de ocratoxina a partir de *Psychotria hoffmannseggiana*. Frutos maduros de *P. hoffmannseggiana* foram coletadas na região da cidade de Passos em área preservada de Cerrado em Minas Gerais, Brasil. Realizou-se diluições decimais e as bactérias isoladas foram caracterizados bioquimicamente e identificadas por seqüenciamento. Para a produção de enzimas, as bactérias foram cultivadas em CMC e MP5 e MP7. Os isolados com melhor produção enzimática foram submetidos ao processo de otimização. A atividade antagonista foi realizada em co-cultura (bactérias e fungos filamentosos) e avaliou-se o crescimento e a esporulação. TLC foram realizados para verificar a produção de micotoxinas. A microbiota predominante foi Gram negativas pertencem à família *Enterobacteriaceae*. Alguns isolados apresentaram potencial para a atividade enzimática e antagonista, especialmente o isolado identificado como *Lysinibacillus fusiformis*. Esta espécie foi melhor produtora de celulases (atividade máxima 103,1 mg glicose $min.^{-1}$ mg^{-1} de proteína). Três dos 105 isolados de bactérias estimularam a produção de micotoxinas. Conclui-se que estes isolados apresentam potencial biotecnológico para a área de agro-indústria e meio ambiente.

Palavras-chave: microrganismo, *Psychotria hoffmannseggiana*, aplicacao biotecnológica.

Introduction

Brazil has great biodiversity, which is observed in the Cerrado biome typical of the tropical Brazilian savanna; the Cerrado biome occupies approximately 23.1% of the country. It is the second largest biome, surpassed only in the Amazon region (KLINK; MOREIRA, 2002). This biome has a great diversity of macro-and micro-organisms. This diversity includes a great richness of fruit species; of particular interest are the fruits with pleasant and even exotic sensory traits, such as color, flavor and aroma.

Few studies have studied the epiphytic microbiota of Cerrado fruits (FERREIRA;

JUNQUEIRA, 2007; TRINDADE et al., 2002) and their biotechnological applications in the agro-industry, such as biocontrol, food industry, textiles, waste treatment and enzyme production by microorganisms.

The epiphitic microbiota can show cellulolytic and pectinolytic enzyme activities (FERREIRA; JUNQUEIRA, 2007; TRINDADE et al., 2002). When excreted by microorganisms, these enzymes cause the depolymerization of pectin and cellulose compounds and the subsequent release of simple sugars, such as glucose. These sugars are used as carbon and energy sources. These microorganisms have the potential to be useful in industrial applications. Microorganisms producing cellulase, for example, have been used in pharmaceuticals, bio-fuels, textiles, pulp and paper and waste treatment (IKEDA et al., 2006; LEE et al., 2008; TANAKA et al., 2006).

Coffee is an important part of Brazilian culture and the national and state economy because of its exportation. Sanitary control is essential for marketing and consumer safety and focuses on the control of mycotoxin-producing fungi. Among the mycotoxins, ochratoxin A (OTA) is the primary mycotoxin associated with coffee beans. OTA is produced by several species belonging to the genera *Penicillium* and *Aspergillus* and nominally in *A. carbonarius*. The control of OTA in food should be instigated before the harvest process to prevent OTA contamination; however, if the contamination persists after this phase, the OTA contamination should be controlled with post-harvest interventions (KAPETANAKOU et al., 2012).

Decontamination methods can be physical, chemical or biological. The biological control methods (biocontrol) that have been examined include bacteria and yeasts in single or mixed cultures (KAPETANAKOU et al., 2012; KONG et al., 2010; MATEO et al., 2010; RAMOS et al., 2010). The antagonistic activity of substances can affect the growth and the sporulation of harmful fungi and, indirectly, affect the mycotoxin level. *Debaryomyces hansenii and Pichia anomala* species isolated from coffee fruit have been shown to be capable of inhibiting or reducing sporulation and consequent toxin production when co-cultivated *in vitro* with *Aspergillus ochraceus*, *Aspergillus parasiticus* and *Penicillium roqueforti* (RAMOS et al., 2010).

The objectives of the present study were to isolate, characterize and identify cellulolytic and pectinolytic bacteria for possible future biotechnological applications and to select bacteria from *Psychotria hoffmannseggiana* in the Cerrado region of Minas Gerais with antagonistic activity against OTA-producing *A. carbonarius* isolated from coffee beans.

Material and methods

Sampling and microbiological analysis

Ripe fruit without injury of *Psychotria hoffmannseggiana* (Willd. ex Roem. & Schult.) Müll. Arg. (popular name Erva de rato) (Figure 1) were collected in the region of Passos City in the preserved Cerrado area in Minas Gerais State, Brazil during the wet season (254.8 mm). The average temperature was higher than 18ºC, and the geographic location was 20º43'08"S and 46º36'35"W.

Two hundred grams of the fruit were transferred to a bottle containing 1800 mL of saline peptone diluent (0.1% of peptone, 0.5 of NaCl, 0.03 $Na_2H_2PO_4$). After 15 min. of mixing, ten-fold dilutions were prepared. Appropriate dilutions were spread on plates of Nutrient Agar (NA) for isolation of easily grown microorganisms and on plates of Nutrient Agar supplemented with Nystatin (400 mg 1000 mL^{-1}) (NA+) for examination of bacteria and fungi inhibition. Eosin Metilieno Blue (EMB) was used to select for Gram-negative coliform bacteria, and Man Rogosa Sharpe (MRS) was used for the cultivation of lactic acid bacteria. All cultives were done on triplicate. Plates were incubated at 30°C for 24-48 hours after which the total count was made and expressed in log CFU g^{-1}. The square root of the number of colonies from each type of media was taken at random for identification (HOLT et al., 1994). Isolates were purified and stored at -80°C in a 20% glycerol concentration.

Figure 1. *Psychotria hoffmannseggiana*. Fruit used for the isolation of bacterial isolates with antagonistic and cellulolytic potential.

Biochemical characterization and cluster analysis

Pure cultures of the various colonial morphotypes were characterized by Gram staining, microscopic morphology, amount of catalase, sporulation, and motility, as described by Holt et al. (1994). Isolates with similar characteristics were selected for PCR amplification and DNA sequencing. The Gram-negative bacteria were examined using Bac Tray system I, II and III.

PCR amplification and DNA sequencing of the 16S rRNA gene

Representative isolates of each REP-PCR profile were selected for amplification of the 16S rRNA gene as described by Pereira et al. (2006). DNA (2 μL) was added to 30 μL of Taq PCR Master Mix (Qiagen, São Paulo, Brazil), 26 μL of H_2O, 1 μL of primer 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1 μL of primer 1512r (5'-ACGGCTACCTTGTTACGACT-3'). The PCR reaction as follows: initial denaturation at 95°C for 10 min.; 25 cycles of 93°C for 1 min., 50°C for 1 min., and 72°C for 1 min. 30 s; and a final elongation at 72°C for 5 min. The presence of PCR products was confirmed by electrophoresis on a 1% agarose gel in 1x TAE buffer at 70 V for 30 min. The gel was stained with SYBR Green (Invitrogen, Foster City, CA, USA) and visualized under a transilluminator. The sequencing of amplicons was performed at Macrogen Inc. (Seoul, South Korea), and the sequences were compared with the GenBank database using the BLAST algorithm (National Centre for Biotechnology Information, Maryland, USA).

Enzyme Evaluation

All isolates were submitted for screening for the production of cellulase, pectin lyase and polygalacturonase. The isolates were reactive in PCA medium, and 10 μL of each isolate suspension was plated in specific media.

The isolates that showed growth in specific media (item 2.4.1. and 2.4.2) were reactivated in PCA medium, and the cells concentrations were standardized at 1.5 x 10^9 cells mL^{-1} using Mc Farland nº5 for the semi-quantitative analysis. The isolates that showed μigher growth than the control were used for the quantitative assays. All reagents used were purchased from Merck, Sigma or Himedia.

Screening for pectinase activity

Pectinolytic activity was determined using the methods described by Schwan et al. (1997) and Hanckin and Lacy (1984). The yeasts and bacteria were grown in media containing the following: 0.5% glucose, 0.5% poligalacturonic acid, 0.6% KH_2PO_4, 0.1% yeast extract, 0.2% $(NH_4)_2SO_4$ and 1.5% agar. The media also contained 0.1 mL of the following solutions: 0.0001% $FeSO_4$, 0.02% $MgSO_4$, 0.0001% $CaCl_2$, 0.0002% H_3BO_3, 0.0002% $MnSO_4$, 0.0014% $ZnSO_4$. $7H_2O$, 0.001% $CuSO_4$. $5H_2O$ and 0.0002% MoO_3. The pH of the media was adjusted to 5.5. Polygalacturonic acid (for MP5 media) was added as a substrate to determine the polygalacturonase (PG) activity, and mineral medium plus pectin was added as a substrate (for MP7 media) to determine the pectin lyase activity (HANCKIN; LACY, 1984). The composition of the MP7 media is the same as the MP5 media except that polygalacturonic acid was replaced by pectin and that the pH was 7.2. Enzyme activity was indicated by the formation of a clear halo around the colonies after precipitating polygalacturonic acid with 1% cetyl trimethyl ammonium bromide (CTAB).

Screening for cellulase activity

The qualitative assessment of the production of cellulases was performed by the method proposed by Kasana et al. (2008). Isolates were considered cellulase-producing if a halo formed around the colony. Isolates with halos less than 1.0 mm were considered weak producers (+). Isolates with halos greater than 1.0 mm were considered strong producers (++). Non-producers (-) were indicated by the absence of a halo.

Quantitative assays of cellulase production

The isolates with a halo greater than 1.0 mm were submitted for quantitative assays.

The quantification of cellulase production was performed in CMC culture medium with varying pHs (5-9), concentrations of inoculum (10^4 to 10^8 CFU g^{-1}) and concentrations of glucose (0.25 to 5%). The endoglucanase activity was measured by an indirect spectrophotometric method using hydrazide p-hydroxybenzoic acid (1% PAHBAH) for measurement of glucose released by enzymatic action (LEVER, 1972). The determination of total proteins was obtained by a Bradford (1976) assay. The units were defined as the quantity of glucose released per minute per milligram of protein.

Co-culture of *Aspergillus carbonarius* with the bacterial isolates

Eighty-six bacterial isolates were co-cultured with the fungus *Aspergillus carbonarius*. For co-cultivation, a standardized bacterial cell suspension (McFarland scale n 1) was spread with a swab on a

plate containing CYA (Czapek Yeast Agar). The spores of *A. carbonarius* were standardized at 10^7spores mL^{-1} and immediately inoculated in media containing the bacterial cell suspension. The control consisted of inoculation of the spore suspension without bacteria. The experiment was conducted in triplicate. The plates were incubated for 7 days at 30°C. The growth inhibition was calculated as the percentage of inhibition of radial growth relative to the control. The presence or absence of the toxin was determined by Thin Layer Chromatography (TLC).

Antagonistic activity of the bacterial extract

The bacterial extract (extract) was obtained after growth of bacterial isolates in NA for 24h at 30°C and centrifuged at 10,000 rpm for 10 minutes. For this assay, the bacterial isolates that showed the strongest inhibition of the growth of *A. carbonarius* (item 2.5) were chosen. Two milliliters of extract obtained from each isolate were homogenized in 18 mL of CYA medium and poured into Petri dishes. After solidification of the culture medium and extract, the spore suspension was inoculated as previously described. The growth inhibition was calculated as the percentage of inhibition of radial growth relative to the control. The presence or absence of the toxin was determined by Thin Layer Chromatography (TLC).

Thin Layer Chromatography (TLC)

After assessing the growth of the fungal colony co-cultured with the different bacteria isolates, circular cuts approximately 25 mm from the mycelium of the fungus were placed on agar plates for Thin Layer Chromatography. The following materials were used: ochratoxin A standard (Sigma-Aldrich), thin-layer chromatography plates (Silica Gel Merk, 60, 20x20), and, for the mobile phase, ethyl acetate, TEF-toluene and formic Acid 90% (50:40:10). After elution, the plates were dried by air flux. Mycotoxin production was confirmed using ultraviolet light with λ 366 nm in a chromatovisor CAMAG (UF-BETRACHTER). The isolates considered to be OTA producers presented a retention factor (RF) and a fluorescence spot similar to that of the OTA standard (FILTENBORG; FRISVAD, 1980).

Results and discussion

Thirty-two different bacterial morphotypes isolated from the fruit *Psychotria hoffmannseggiana* were observed, totaling 105 isolates. The epiphytic microbial population of the fruit varied from 1.76 x 10^4 to 2.1 x 10^5 CFU g^{-1}. The species isolated in this fruit are described in Table 1.

Of all the isolates (105), 41% were *Enterobacter aerogenes*, 32% were *Klebsiella oxitoca*, 11% were *Tatumella ptyseos,* 5.5% were *Hafnia alvei*, 3.5% were *Shigella* sp and 7% were *Lysinibacillus fusiformis*. The predominance of Gram-negative bacterial isolates, especially of the *Enterobacteriacea* family, was consistent with that observed in banana-associated microbes in Uganda (ROSSMANN et al., 2012). The fruit were sampled during the wet summer season, which is characterized by high rainfall and high temperature (higher than 250 mm and 20ºC, respectively). The species that dominate the microbiota were found to be different between the wet and dry seasons, as was also observed previously by Silva et al. (2000) in coffee fruits. The same was observed by Peñuelas et al. (2012) in the microbiota associated with *Quercus ilex*; changes in the climate likely significantly altered the microbial abundance and composition of the phyllosphere. Gram-negative bacteria represent an important bacterial group because some species are pathogenic, as found in this study. As of yet, *Psychotria hoffmannseggiana* fruit rarely used as food for the local population. For biotechnological applications, the microorganisms should be recognized as GRAS, and this explains why some species of the *Enterobacteriacea* family are not utilized.

Table 1. Incidence of species found in *Psychotria hoffmannseggiana*, a typical fruit of savanna like in Brazil.

SPECIE	ISOLATES	POPULATION (log UFC g^{-1})
Klebsiella oxytoca	UFLABIO 02.2; 07.1; 07.2; 08.1; 09; 13; 14.2; 15.1; 15.2; 16.1; 16.2; 17.1; 17.2; 23.1; 29; 68.2; 80.2.1; 83.1.1	3.47 to 4.84
Enterobacter aerogenes	UFLABIO 01; 03.1; 03.2; 04.1; 10.1; 10.2; 11.1; 11.2; 12.1; 12.2; 33.1; 33.2; 34.1; 39; 40; 49; 50; 51.1; 51.2; 57; 82.1	4.0 to 4.43
Lysinibacillus fusiformis	UFLABIO 05.1; 06.1; 06.2; 35.1.1	3.3
Hafnia alvei	UFLABIO 31.2; 32.3.1; 32.3.2	3.3
Tatumella ptyseos	UFLABIO 42; 43; 44; 45; 46; 47	4.6
Shigella sp	UFLABIO 59; 60	3.6
Gram negative not identified	UFLABIO 05.2; 36.2; 37; 41; 48; 52; 53; 61; 62; 69; 78;	3.7
Gram positive not identified	UFLABIO 18.2;	3.48

* Similarity less than 97% (Genbank database).

Evaluation of enzyme production

The 105 isolates from *P. hoffmannseggiana* were tested for pectinase and cellulase production. It was observed that 32% of the isolates did not show the capacity to produce cellulolytic or pectinolytic enzymes in the conditions tested, and 24% produced cellulase or pectinase enzymes. The majority of isolates that produced enzymes produced cellulase (31%). These microorganisms can promote the infection of the plant because cellulase and pectinase are necessary for infection. The primary infection requires pectinolitic action and, subsequently, cellulase action. Thus, some microorganisms observed in this study can act as secondary colonizers (ISAAC, 1992) because they only produce cellulase. An additional test with inoculum standard (Scale Mac farland n.5) was performed on forty-six isolates. The results of these semi-quantitative analyses are presented in Table 2. The pectinase production was lower than the cellulase production. Two of the isolates tested showed a higher capacity for secreting cellulase and were selected for the quantitative assays. These strains were identified after sequencing as *Lysinibacillus fusiformis.* Some genera of bacteria mentioned in the literature that have cellulolytic activity include: *Bacillus, Clostridium, Cellomonas, Rumminococcus, Alteromonas, Acetivibrio, Bacteriodes* (ROBSON; CHAMBLISS, 1989) and *Vibrio* (GAO et al., 2010). Several species in the *Bacillus* genus have been described as producers of cellulase, including *Bacillus* sp. (MAWADZA et al., 2000), *Bacillus amyloliquefaciens* (LEE et al., 2008) and *Bacillus subtilis* (KIM et al., 2009). However, despite the fact that the *Bacillus* genus is closely related to the *Lysinibacillus* genus, there are no studies reporting the production of cellulase by *Lysinibacillus*.

For the assay of cellulase production, an experimental model was used that allowed the culture parameters (pH 5-9, concentration of inoculum 10^4 to 10^8 CFU g^{-1} and concentration of glucose 0.25 to 5%) to be varied simultaneously in the CMC culture medium. Following cultivation of the bacterial isolates, the enzymatic activity and biomass production were evaluated.

The 13 experiments carried out on the 2 isolates (Table 3) showed that the UFLABIO 35.1.1 isolate produced the highest enzymatic activity (103.1 mg glucose $min.^{-1}$ mg^{-1} protein) in 72 hours of culture (culture conditions were pH 6, 10^7CFU g^{-1} and glucose concentration of 0.5%). A pH of 6 was optimal for the *Pseudomonas* sp based on lignocellulosic material (CHENG; CHANG, 2011); however, previous studies showed that the optimal pH for sporulated-bacteria producing cellulase is higher than 8 (TRIVEDI et al., 2011; KORPOLE et al., 2011). Before 72h of culture, it was not possible to detect enzyme activity in the UFLABIO 35.1.1 isolate. Therefore, it is necessary to extend the culture time for future studies.

In contrast, the lack of enzyme activity and increased biomass in some assays (numbers 5 to 9) were most likely due to higher concentrations of glucose in the culture medium. Glucose can inhibit cellulase production because it is used by the bacteria as a carbon and energy source and consequently increases the biomass. These results confirm the repressive action of glucose at concentrations above 1% on the synthesis of enzymes by filamentous fungi that was previously proposed (GOYAL et al., 1991).

Table 2. Semi-qualitative enzymatic test by some isolates bacteria from *Psychotria hoffmannseggiana*. CMC- carboxymetilcellulase, PG: polygalacturonase, PL: Pectin Lyase. In bold the better producer of cellulase.

Species	Halo (mm)		
	CMC	PG	PL
Enterobacter aerogenes (UFLABIO 49)	0	0	0.3
Enterobacter aerogenes (UFLABIO 49)	0	0	0.3
Enterobacter aerogenes (UFLABIO 57)	0	0	0.3
Hafnia alvei (UFLABIO 46)	0	0	0.2
Klebsiella oxytoca (UFLABIO 16.1)	0	0	0.3
Klebsiella oxytoca (UFLABIO 17.1)	0	0.1	0
Klebsiella oxytoca (UFLABIO 23.1)	0.2	0	0
Klebsiella oxytoca (UFLABIO 84.2)	0	0	0.5
Klebsiella oxytoca (UFLABIO 84.2)	0	0	0.5
Klebsiella oxytoca (UFLABIO17.2)	0.2	0	0.3
Lysinibacillus fusiformis (UFLABIO 18.2)	1.3	0	0.2
Lysinibacillus fusiformis (UFLABIO 35.1.1)	2.1	0.4	0.8
Lysinibacillus sp (UFLABIO 35.1.2)	0	0	0.2
Shigella sp (UFLABIO 59)	0	0	0.4
Tatumella pytseos (UFLABIO 46)	0	0	0.2

Antifungal activity

The best way to prevent mycotoxin production is adopting good agricultural practices during the pre-harvest. However, when mycotoxin has occurred it is necessary carry out detoxification methods (KAPETANAKOU et al., 2012).

Of the 105 isolates from *P. hoffmannseggiana*, 84 were tested for antifungal activity. In the first assay, the bacteria were spread on superficial media and were inoculated immediately after spore suspension, allowing direct contact between the bacteria and the *A. carbonarius.* The bacterial isolates that showed an antagonistic effect were identified after sequencing as *Enterobacter aerogenes* (pathogenic), *Lysinibacillus fusiformis*, *Klebsiella oxytoca* and *Hafnia alvei*. *Tatumella ptseos* (UFLABIO 42, 43, 45 and 46) and *Shigella* sp (UFLABIO 59) did not show any antagonistic activity; however, some isolates of these species showed antagonistic activity. These physiological differences are related to the strain of bacteria and the concentration of mycotoxin (MATEO et al., 2010).

It is interesting that 67.8% of the isolates evaluated had antifungal activity (Table 4). Some isolates only showed antagonistic activity over fungal growth (4 isolates) and no effect over mycotoxin production. However, the majority of isolates had an inhibitory effect on mycotoxin production. Most of the microorganisms with antagonistic activity were isolated from the fruit surface, fruit plant and soil (JANISIEWICZ; KORSTEN, 2002).

Table 3 Design experimental model used for quantitative assay for cellulase production by bacteria isolates from fruit Cerrado (Brazil), enzymatic activity and the biomass production after 72h of period incubation.

Assay	pH	Inoculum concentration (CFU g^{-1})	Glucose concentration (%)	Cellulase activity		Biomassa (mg)	
				UFLABIO 18.2	UFLABIO 35.1.1	UFLABIO 18.2	UFLABIO 35.1.1
1	6	10^5	0,5	12.46	0	276	348
2	8	10^5	0.5	4.21	49.79	247	296
3	6	10^7	0.5	0	103.10	354	239
4	8	10^7	0.5	0	62.94	320	302
5	6	10^5	3.0	0	0	606	511
6	8	10^5	3.0	0	0	413	467
7	6	10^7	3.0	0	0	408	478
8	8	10^7	3.0	0	0	747	503
9	5	10^6	1.0	0	0	655	478
10	9	10^6	1.0	0	0	440	301
11	7	10^6	1.0	0	0	335	391
12	7	10^6	1.0	0	0	373	398
13	7	10^6	1.0	0	0	408	474

Table 4. Bacterial isolate (57 isolates of total) that showed inhibition of OTA production (In OTA) growth (In G) of the *Aspergillus carbonarius*. Symbols (+) represents effect antagonist and (–) no effect antagonist. Isolates (*) means stimulation of mycotoxin production.

Isolated	In OTA	In G	Isolated	In OTA	In G	Isolated	In OTA	In G
UFLA BIO 01	+	+	UFLA BIO 18.2	+	+	UFLA BIO 56	+	-
UFLA BIO 02.1	+	-	UFLA BIO 23.1	+	+	UFLA BIO 57	+	+
UFLA BIO 02.2	+	+	UFLA BIO 29	+	+	UFLA BIO 59	-	-
UFLA BIO 03.1	+	-	UFLA BIO 31.2	+	+	UFLA BIO 60	-	+
UFLA BIO 03.2	+	-	UFLA BIO 32.3.1	-	-	UFLA BIO 61	+	+
UFLA BIO 04.1	+	-	UFLA BIO 32.3.2	-	-	UFLA BIO 62	+	+
UFLA BIO 04.2	+	-	UFLA BIO 33.1	+	+	UFLA BIO 68.2	-	-
UFLA BIO 05.1	+	-	UFLA BIO 33.2	+	+	UFLA BIO 69	-	-
UFLA BIO 06.1	+	-	UFLA BIO 34.1	+	-	UFLA BIO 78	-	-
UFLA BIO 06.2	+	-	UFLA BIO 35.1.1	+	+	UFLA BIO 80.2.1	-	-
UFLA BIO 07.1	+	-	UFLA BIO 35.1.2	-	-	UFLA BIO 82.1	-	+
UFLA BIO 07.2	+	-	UFLA BIO 36.2	-	-	UFLA BIO 83.1.1	-	-
UFLA BIO 08.1	+	+	UFLA BIO 37	-	-	UFLA BIO 56	+	-
UFLA BIO 08.2	+	+	UFLA BIO 39	-	-	UFLA BIO 57	+	+
UFLA BIO 09	+	-	UFLA BIO 40	-	-	UFLA BIO 59	-	-
UFLA BIO 10.1	+	-	UFLA BIO 41	+	+	UFLA BIO 60	-	+
UFLA BIO 10.2	+	+	UFLA BIO 42	-	-	UFLA BIO 61	+	+
UFLA BIO 11.1	+	+	UFLA BIO 43 *	-	-	UFLA BIO 62	+	+
UFLA BIO 11.2	+	-	UFLA BIO 44	+	-	UFLA BIO 68.2	-	-
UFLA BIO 12.1	+	-	UFLA BIO 45 *	-	-	UFLA BIO 69	-	-
UFLA BIO 12.2	+	-	UFLA BIO 46	-	-	UFLA BIO 78	-	-
UFLA BIO 13	+	-	UFLA BIO 47	+	+	UFLA BIO 80.2.1	-	-
UFLA BIO 14.2	+	+	UFLA BIO 48	+	-	UFLA BIO 82.1	-	+
UFLA BIO 15.1	+	+	UFLA BIO 49	-	-	UFLA BIO 83.1.1	-	-
UFLA BIO 15.2	+	+	UFLA BIO 50 *	-	-			
UFLA BIO 16.1	+	+	UFLA BIO 51.1	+	-			
UFLA BIO 16.2	+	+	UFLA BIO 51.2	-	-			
UFLA BIO 17.1	-	-	UFLA BIO 52	+	+			
UFLA BIO 17.2	+	-	UFLA BIO 53	-	-			
UFLA BIO 18.1	+	-	UFLA BIO 54	+	-			

In contrast, some bacterial isolates did not influence fungal growth or mycotoxin production (27 isolates). In some cases, it was observed that the mycotoxin production was stimulated in the presence of bacteria compared to the control (Figure 2). This may be explained by a decrease in the pH of the media due to compounds produced by the bacteria during the cultivation period (7 days). Low pH stimulates mycotoxin production.

Twenty-five percent of the isolates in the co-culture assay showed a reduction in the growth rate of *A. carbonarius* compared to the control. A decrease in the spore production was also observed; however, it is not possible to determine the percentage of reduction in spore production because it was only a visual observation (Figure 2a). Nevertheless, this observation is interesting because the inside of the spore may contain mycotoxins. When the production of spores was reduced, toxin production was also inhibited, as determined by thin layer chromatography (TLC) (Figure 2b). A decrease in spore production translates into lower levels of toxin present in food because some of the mycotoxins accumulate in the spores (RAMOS et al., 2010).

Of all the isolates tested, approximately 61.9% were capable of inhibiting toxin production; however, we were unable to determine the exact mechanism of action. Bacteria, such as *Bacillus*, are capable of absorbing or degrading mycotoxins. It is also possible that mycotoxin production was inhibited by the bacteria.

The second assay was performed on the media extracted after cultivation of each bacterial isolate (a cell-free assay). None of the bacterial media extracts showed antagonistic activity against growth or mycotoxin

production, which indicates the absence or low concentration of bacterial molecules with inhibition potential. This result was different from that observed by Kong et al. (2010) while studying the action of *Bacillus megaterium* against *Aspergillus flavus*. They observed that the metabolites produced by bacteria had a higher antagonistic effect than living bacteria.

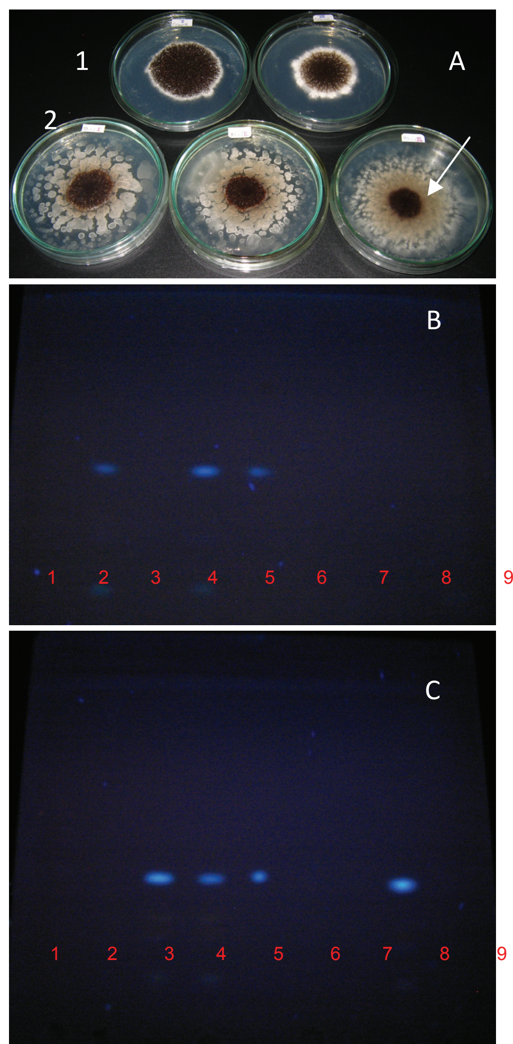

Figure 2. A) Photographs of co-cultures with 4 days of incubation. 1) control, *A. carbonarius* incubated without bacteria, 2) *A. carbonarius* grown with the *Lysinibacillus fusiformis* UFLABIO 35.1.1 isolate. The change of color in a colony indicated the absence of spores (arrow). B) Thin layer chromatography (TLC) mycotoxin analyses showed inhibition of mycotoxin production by the isolate UFLABIO 15.1 (number 1); UFLABIO 18.2 (number 3); UFLABIO 23.1 (number 6); UFLABIO 29 (number 7); UFLABIO 31.2 (number 8) and UFLABIO 35.1.1 (number 9). UFLABIO 17.1 (number 2) showed weak mycotoxin production; Control (number 4); Standard (number 5) **C**) TLC showed stimulation of mycotoxin production by isolate UFLABIO 45 (number 3) and UFLABIO 50 (number 8). Other isolates: UFLABIO 41 (number 1); UFLABIO 44 (number 2); Control (number 4); Standard (number 5); UFLABIO 47 (number 6); UFLABIO 48 (number 7); UFLABIO 51 (number 9).

Conclusion

Psychotria hoffmannseggiana was shown to be naturally colonized by *Enterobacter aerogenes*, *Klebsiella oxytoca*, *Hafnia alvei*, *Tatumella ptseos*, *Lysinibacillus fusiformes* and other bacteria genus. The predominant population consisted of Gram-negative bacteria, and some Enterobacteriacea are pathogenic. The UFLABIO 35.1.1 isolate had the highest enzyme activity (103.1 mg glucose $min.^{-1}$ mg^{-1} protein) has the potential for biotechnological material degradation through cellulolysis. However, a longer cultivation time is necessary to increase yield. With regards to the antagonistic activity, 67.8% of the isolates evaluated inhibited growth and/or mycotoxin production of *Aspergillus carbonarius*; these isolates could be used as biocontrol agents.

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