Food Control 85 (2018) 392–399

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Short communication

Antifungal and aflatoxin-reducing activity of extracellular compounds produced by soil *Bacillus* strains with potential application in agriculture

M.L. González Pereyra [a, b], M.P. Martínez [a, c], G. Petroselli [b, d, e], R. Erra Balsells [b, d, e], L.R. Cavaglieri [a, b, *]

[a] Departamento de Microbiología e Inmunología, Facultad de Ciencias Exactas, Fisico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Ruta N 36 Km 601, 5800 Río Cuarto, Córdoba, Argentina
[b] Member of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina
[c] Fellow of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina
[d] Universidad de Buenos Aires, Facultad de Ciencias Exactas y Naturales, Departamento de Química Orgánica, Pabellón II, 3er P., Ciudad Universitaria, 1428 Buenos Aires, Argentina
[e] CONICET, Universidad de Buenos Aires, Centro de Investigación en Hidratos de Carbono (CIHIDECAR), Facultad de Ciencias Exactas y Naturales Pabellón II, 3er P., Ciudad Universitaria, 1428 Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 15 May 2017
Received in revised form
21 September 2017
Accepted 18 October 2017
Available online 21 October 2017

Keywords:
Aflatoxin B_1
Aspergillus parasiticus
Bacillus
Lipopeptides

ABSTRACT

Toxigenic *Aspergillus flavus* and *A. parasiticus* fungal strains can contaminate a wide variety of food crops with the subsequent production of aflatoxins (AFs) resulting in severe economic losses and public health issues. Biological control is a promising approach to manage AFs contamination in pre- and post-harvested crops. In the present study, the effect of soil-borne *Bacillus* spp. strains on aflatoxigenic *A. parasiticus* growth and AFs production was evaluated and the culture supernatant of the most effective strain was evaluated for the presence of antifungal lipopeptides. Six *Bacillus* spp. strains were able to reduce *A. parasiticus* growth rate significantly ($p < 0.05$). *Bacillus* spp. RC1A was able to inhibit fungal growth almost completely, reducing growth rate to 0.16 mm/h and increasing Lag phase duration (31.72 h) ($p < 0.0001$). RC1A could also reduce AFB_1 concentration produced by *A. parasiticus* ($p < 0.0001$). Organic solvent extraction and chromatographic analysis of RC1A culture supernatant showed the presence of bands corresponding to three of the main groups of lipopeptides (surfactin, iturin A and fengycin) at the expected retention factor (Rf) values; they were also confirmed by MALDI-MS analysis. These fractions were able to inhibit *A. parasiticus* growth and AFB_1 production to non-detectable levels when tested separately in liquid culture media. The further study of the antifungal compounds produced by these strains will determine their potential use to manage AFs contamination in crops and feeds.

1. Introduction

Aspergillus section *Flavi* (especially *A. flavus* and *A. parasiticus*) can contaminate a wide variety of food crops including maize, cottonseed, peanuts, and tree nuts. The subsequent production of aflatoxins (AFs) in susceptible crops results in severe losses for growers every year. Aflatoxins, especially aflatoxin B_1 (AFB_1), are extremely toxic secondary metabolites with carcinogenic, mutagenic and teratogenic effects (IARC, 2002; Zhang, Shi, Hu, Cheng, & Wang, 2008).

Many strategies have been investigated to manage AFs contamination in crops. Biological control appears to be the most promising approach to control AFs in both pre- and post-harvested crops. Bacterial strains with the ability to produce antifungal substances have been tested mainly to control post-harvest fungal contamination. Many *Bacillus* strains (especially *B. subtilis, B. amyloliquefaciens* and *B. circulans*) are known to suppress fungal growth *in vitro* due to the production of antifungal antibiotics especially the

* Corresponding author. Departamento de Microbiología e Inmunología, Universidad Nacional de Río Cuarto, Ruta 36 Km, 601, 5800 Río Cuarto, Córdoba, Argentina.
E-mail addresses: mgonzalezpereyra@exa.unrc.edu.ar (M.L. González Pereyra), mmartinez@exa.unrc.edu.ar (M.P. Martínez), lcavaglieri@exa.unrc.edu.ar (L.R. Cavaglieri).

https://doi.org/10.1016/j.foodcont.2017.10.020
0956-7135/

non-ribosomally synthesized cyclic lipopetides of the surfactin, iturin and fengycin families (Afsharmanesh, Ahmadzadehb, Javan-Nikkhahb, & Behboudib, 2014; Caldeira, Santos Arteiro, Coelho, & Roseiro, 2011; Cho et al., 2009, 2003; Das, Mukherjee, & Sen, 2008; Gong et al., 2015; Ji et al., 2013). Lipopeptides are amphiphilic membrane-active biosurfactants and peptide antibiotics with potent antifungal activities which can be used as biopesticides for plant and post-harvest protection. Moreover, lipopeptides are easily biodegradable in soils, constituting a healthier and environmentally friendly alternative to synthetic fungicides (Caldeira et al., 2011). Although the use of synthetic fungicides is the most effective treatment to control fungal disease, there is an urgent need to find equally effective but safer means of controlling post-harvest fungal pathogens, mainly due to the toxicity of the synthetic fungicide residues exert on human health and the environment (Droby, 2006).

In the present study, the effect of soil-borne *Bacillus* spp. strains on aflatoxigenic *A. parasiticus* growth and AFs production was evaluated and the culture supernatant of the most effective strain was evaluated for the presence of lipopeptides.

2. Materials and methods

2.1. Strains

Thirteen *Bacillus* strains were obtained from lake sediment and soil samples according to methodology described by Sosa-Pech et al. (2012). Strains were cultivated in nutrient agar (NA) plates and morphological traits (colony morphology, color, size, shape, elevation, margins) Gram stain and catalase test were recorded after 24 h incubation at 28 °C. Spore formation was observed in malachite green stained slides of 72 h cultures. Strains showing *Bacillus* genus' characteristics (Gram-positive, rod-shaped, spore forming bacilli) were maintained in NA slants and three colonies of each strain were inoculated in microtubes containing 0.2% glycerol and stored at −80 °C for future studies. *Bacillus* species were identified using a MALDI-TOF mass spectrometer (Bruker Daltonics MALDI Biotyper) using single colonies of 24 h cultures of each strain. Three strains were identified as *B. mojavensis*, two as *B. subtilis*, five as *B. cereus*, one as *B. megaterium* and two as *B. mycoides*.

2.2. Influence of Bacillus strains on Aspergillus parasiticus growth and aflatoxin production

Malt extract agar (MEA) dishes amended with two ml of 24 h *Bacillus* cultures (10^8 cells/ml) and MEA control dishes were inoculated with a central spot of a *A. parasiticus* NRRRL 2999 conidial suspension in 0.2% soft agar (10^3 cells/ml) and incubated at 25 °C until the colony on the control plates reached the edge of the dish. The diameter of growing fungal colonies was measured in two directions at 90° from each other to obtain the mean diameter for each colony. The growth rate (mm/h) was calculated by linear regression of colony diameter against time during the linear phase of growth for each set of conditions tested. The linear section of the graph was extrapolated to a zero increase in diameter (i.e. 5.0 mm diameter) and the intercept on the time axis was defined as the Lag phase (hours in which the colony reaches 5.0 mm of diameter). Three agar plugs were taken from each plate and AFs accumulated in the culture media were extracted with chloroform according to Geisen (1996) with some modifications and the extracts were analyzed for AFB_1 by HPLC according to Trucksess, Stack, Nesheim, Albert, and Romer (1994). Briefly, AFs were extracted from agar plugs with 1 ml chloroform by centrifugation at 8000 rpm. The organic layer (800 μl) containing the toxin was collected, filtered and evaporated to dryness under N_2 stream. Samples were redissolved in 400 μl mobile phase and 200 μl aliquots of were derivatized with 700 μl trifluoroacetic acid: acetic acid: water (20:10: 70, v/v) solution. Fifty μl aliquots were injected in a Waters Alliance 2695 system coupled to a fluorescence detector (Waters 2487). Chromatographic separations were performed on stainless steel, C18 reversed phase column (Luna Phenomenex, 150 × 4.6 mm id., 5 μm particle size). Water (4 v/v): methanol (1 v/v): acetonitrile (1 v/v) was used as mobile phase at a flow rate of 1.5 ml min^{-1} and the limit of detection (LOD) was 0.5 ng/ml. The fluorescence of AFB_1 derivatives was recorded at excitation and emission wavelengths of 360 and 460 nm, respectively. A calibration curve was constructed by injecting AFB_1 standards of 5; 30 and 50 ng/ml and quantification of the toxin levels in samples were calculated by comparison of peak areas. The AFB_1 standards solutions were prepared according to AOAC (1995). The experiment was repeated three times (three replicates per strain) and the results were obtained comparing average values ± standard error (SE).

2.3. Extraction of the antifungal compounds

Bacillus mojavensis RC1A was selected and the cell-free culture supernatant (CFCS) was obtained by centrifugation (8000 rpm, 15 min at 4 °C) and filtration (0.22 μm cellulose nitrate filters) of a 24 h culture in nutrient broth. The extraction of the antifungal compounds was performed according to Ji et al. (2013). Briefly, the CFCS was mixed with equal volume of hexane, chloroform, ethyl acetate, and n-butanol, successively. Each fraction was collected, concentrated and dissolved with methanol. The crude extract from the butanol layer was dried to remove methanol *in vacuo* and separated by silica gel column chromatography (70–230 mesh; Merck, Darmstadt, Germany) with chloroform:methanol (20:1), chloroform:methanol (10:1), chloroform:methanol (5:1) and chloroform:methanol (2:1) as mobile phase. All fractions were collected and a preparative thin layer chromatography was made to separate compounds in the extracts (especially lipopeptides) and an aliquot of each was stored at −20 °C for antifungal activity testing. The plates were developed in chloroform:methanol:H_2O (65:25:4, v/v). The chromatograms were air-dried and compounds revealed under UV light (265 and 360 nm) by spraying with water. The different fractions were separated by scraping the silica from the TLC plates, compounds were extracted with chlorophorm:methanol (2:1, v/v) and the extracts were tested for antifungal activity against *A. parasiticus* NRRL 2999. An aliquot of each extract was taken to confirm lipopeptides presence by HPLC in further studies. Lipopeptides were also extracted from CFCS using n-butanol by an alternative methodology described by Afsharmanesh et al. (2014). After separation and complete evaporation of the butanol layer, the remaining residue was dissolved in methanol. This methanolic fraction was separated by thin layer chromatography (TLC) on normal-phase HPTLC silica gel 60 F254 aluminum sheets (Merck, Darmstadt, Germany) and developed with chloroform:methanol:water (65:25:4, v/v). Lipopeptides were visualized by spraying distilled water onto the TLC sheets and their corresponding retention factor (Rf) values were estimated (Razafindralambo et al., 1993) and compared with characteristic Rf values of lipopeptides obtained in other studies (Afsharmanesh et al., 2014).

2.4. Antifungal activity

The antifungal activity against *A. parasiticus* NRRL 2999 of all the fractions obtained was tested as described by Palumbo, Baker, and Mahoney (2006). Sterile 48-well plates containing 1 ml yeast extract saccharose (YES) broth per well were added 50 μl of the