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Effect of *Bacillus spp*. on *Aspergillus westerdijkiae* growth, sporulation and ochratoxin A production in green-coffee medium

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Abstract

Aspergillus westerdijkiae is one of the most important spoilage and toxigenic fungi contaminating coffee beans and may produce ochratoxin A (OTA), a mycotoxin that characterize a health risk to the coffee consumers. Biological control strategies can be used for prevention of fungal invasion and decrease mycotoxin exposure. The aims of this study were to evaluate the *in vitro* effect of three *Bacillus* sp. biocontrol candidates on *A. westerdijkiae* mycelial growth, spore counts and OTA production. A green-coffee based medium was inoculated with *A. westerdijkiae* and *Bacillus* spp. (*B. safensis* RF69, *B. amyloliquefaciens* RP103 and *B. subtilis* RP242) and after incubation, the fungal growth, sporulation and mycotoxin production was evaluated. Mycelial growth rate was reduced in a range between 76-95% and conidial production was also significantly decreased. All isolates were capable of reducing OTA production in a range between 62-96%. The results showed that the biocontrol candidates may be an effective control method for *A. westerdijkiae* and OTA in coffee.

Keywords: biological control; food mycology; mycotoxins; fungi; food safety.

Practical Application: Intense inhibition of *A. westerdijkie* by *Bacillus* spp. in a coffee-based medium.

1 Introduction

Coffee is the most consumed beverage worldwide, with the estimative of 600 billion cups served every day. Brazil is the largest coffee producer and exporter, being responsible for producing more than 43 million sacs in 2015 and having approximately 2.25 million hectares destined to this culture (Associação Brasileira de Indústria de Café, 2016). The quality of the coffee beans affect directly on its price on the market and may have profound economic impacts when those grains are infested with spoilage agents such as filamentous fungi.

Several fungi can contaminate the coffee beans and apart of causing direct spoilage, different toxins may be produced. Ochratoxin A (OTA) is one of the major mycotoxins found in coffee worldwide (Ostry et al., 2013; Taniwaki et al., 2014; Geremew et al., 2016). This mycotoxin has nefrotoxic, teratogenic and mutagenic in animals and is considered by the International Agency of Research on Cancer (IARC) as Group 2B: Possibly carcinogenic to humans (IARC, 2002, 2012). On warm climates such as those found in Brazil, OTA contamination in coffee is mainly associated with *Aspergillus* section *Circumdati* members *A. steynii* and *A. westerdijkiae*, with the latter being reported as the main responsible of OTA production in this product by several studies (Gil-Serna et al., 2011; Ostry et al., 2013; Taniwaki et al., 2014; Geremew et al. 2016). *Aspergillus westerdijkiae* is an opportunistic fungus that may grow and produce OTA under a different set of water activities and temperatures and can invade coffee beans both at pre-harvest and during storage, characterizing this species as a flexible contaminant (Gil-Serna et al., 2014).

Traditionally, fungal control during pre-harvest is mainly conducted by synthetic fungicide applications, but due to the adverse effects caused by those products on the environment and the consumers, alternative ecological-friendly methods are being employed to prevent fungal contamination. Biological control is nowadays broadly used as a prophylactic method for phytopatogens control on several agricultural cultures (Ongena & Jacques, 2008; Pérez-García et al., 2011; Chulze et al., 2014).

Among different biocontrol agents used worldwide, soil bacteria from *Bacillus* genus present promising characteristics such as low nutritional requirements, resistance to adverse environmental conditions, low or none toxicity to environment and the production of a large number of antifungal compounds (Ongena & Jacques, 2008; Pérez-García et al., 2011). Although species from *Bacillus* genus are already being used as biocontrol agents to a diverse number of phytopathogens and contaminants, its efficacy towards *Aspergillus westerdijkiae* growth and OTA production remains commercially unexplored. Therefore, the aims of this study were to evaluate the effect of the interaction with three *Bacillus* spp. on *A. westerdijkiae* mycelial growth rate and conidial and OTA production in a green-coffee based medium.

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2 Materials and methods

2.1 Bacterial and fungal isolates

Three *Bacillus* sp. isolates previously selected on our laboratory were used in this trials: *Bacillus safensis* RF69; *Bacillus amyloliquefaciens* RP103 and *Bacillus subtilis* RP242. One isolate of OTA producer *A. westerdijkiae* previously isolated from coffee beans and made available by ITAL (Food Technology Insitute, Campinas, Brazil) was also used.

2.2 Inoculum preparation and culture media

The assays were performed in green-coffee medium as proposed by Gil-Serna et al. (2014), which contains 3% (w/v) of green-coffee beans and 20 g L^{-1} bacteriological agar (Kasvi). The medium preparation was conducted by boiling 30 g of grounded green-coffee beans on 1 L of distilled water for 30 mins with subsequent filtration of the mixture and re-adjustment of the volume to 1 L of distilled water. For inoculum preparation, bacterial isolates were grown for 24h on 523 broth (Kado & Heskett, 1970) and cell concentrations were adjusted for 10^9 CFU mL^{-1} by serial dilutions. *A. westerdijkiae* isolate was grown on Malt Extract Agar (MEA; Merck) at 25 °C for 7 days and the colonies were used for removal of agar plugs (5 mm) from the border of growing colonies with a cork borer (Pitt & Hocking, 2009).

2.3 Effect on fungal mycelial growth rate

For the evaluation of the effect of *Bacillus* sp. isolates on *A. westerdijkiae*'s mycelium growth rate, we used the method proposed by Bluma & Etcheverry (2006). Briefly, aliquots (1 mL) of bacterial suspensions were inoculated on Petri plates (final concentration of 10^9 cells mL^{-1}) and after that, 19 mL of melted Green-Coffee Based Medium (GCM) was inoculated and homogenized. After solidification, agar plugs of *A. westerdijkiae* mycelium were inoculated in the center of the plate.

Treatment and control plates (*A. westerdijkiae* growing alone) were incubated on polyethilene bags for 10 days at 25 °C in triplicates. This assay was repeated three times. Fungal growth in diameter, was evaluated daily by measuring two radii at right angles one to another with a ruler (Cavaglieri et al., 2005; Bluma & Etcheverry, 2006). The radial growth rate (cm day^{-1}) was calculated by linear regression.

2.4 Effect on conidial production

The ten-day cultures described on item 2.3 were also used to evaluate the effect of interaction with *Bacillus* spp. on *A. westerdijkiae* conidial production. Cultures were washed twice with 5 mL of peptone water (0.01%) and harvested three times using a glass handle.

Conidial counts of the 10 mL suspensions were performed using a Neubauer chamber and the number of conidia mL^{-1} was calculated. Subsequently, total conidial number was calculated and determined the concentration of conidia per cm^2 of mycelium. This evaluations were repeated three times for validation of the obtained results.

2.5 OTA extraction and quantification

Ochratoxin A extraction from the cultures were conducted by crumpling the whole cultures (mycelia and media) on 10 mL of chloroform twice and subsequently filtering using Whatman filter paper and evaporated to dryness using gaseous nitrogen. The dried extracts were resuspended in 250 µL of the initial mobile phase (Acetonitrile:H_2O:formic acid, 10:90:0.2%), transferred to 10 µL vials and injected on Ultra-Performance Liquid Chromatography and Sequential Mass Spectometry (UPLC-MS/MS; Waters Acquity UPLC system coupled with Waters Quattro PremierTM XE Mass Spectometer) system as described by Castro et al. (2014).

2.6 Statistical analyses

One-way ANOVA was applied to data analyzes, using Statistica 10.0 (StatSoft, Inc.) software for Windows. Tuckey test was conducted for treatments mean comparison using $p<0.05$ significance and linear regression analyzes were conducted to calculate fungal mycelial growth rate.

3 Results and discussion

Results of the effect of interaction with *Bacillus* isolates on *A. westerdijkiae* mycelial growth rate and conidial and OTA production are showed on Table 1. The fungal diameters per day are showed on Figure 1.

All three *Bacillus* sp. isolates were able to significantly ($p<0.05$) reduce *A. westerdijkiae* mycelial growth in a range between 76 and 96%. Growth per day was significantly reduced by all *Bacillus* isolates, which *B. amyloliquefaciens* RP103 and *B. subtilis* RP242 showed to be more effective and stable in restraining *A. westerdijkiae* propagation than *B. safensis* RF69, that allowed a small growth progression though the incubation period (Figure 1). The ability to produce conidia was also negatively affected by all *Bacillus* isolates, which significantly reduced the conidia per cm^2. While direct antagonism has already been reported by Petchkongkaew et al. (2008), this is the first study that show reduction of *A. westerdijkiae* mycelial growth rate and the reduction of the conidial production by *Bacillus* spp. Inhibition of growth rate often lengthens fungal

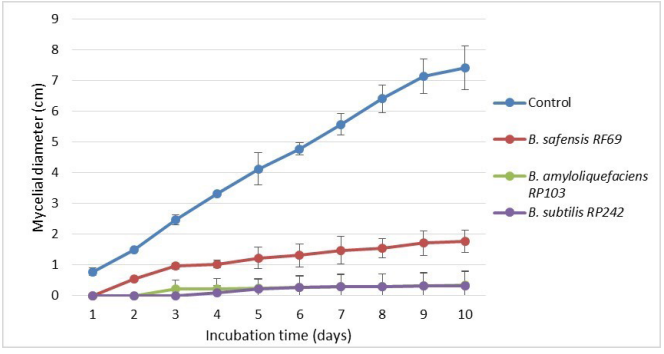

Figure 1. *Aspergillus westerdijkiae* mycelial diameter per day when interacting with biological control agents *Bacillus safensis* RF69, *B. amyloliquefaciens* RP103 and *B. subtilis* RP242 in a Green-Coffee Based medium.

Table 1. Effect of interaction of *Bacillus* isolates and *Aspergillus westerdijkiae* on fungal mycelial growth rate and conidial and ochratoxin A production.

	Colony diameter (cm)	Mycelial growth rate (cm day^{-1})	Mycelial growth reduction (%)	Conidial production (log conidia per cm^2)	Ochratoxin A production (μg kg^{-1})	OTA production reduction (%)
Control	7.43 ± 0.2 a	0.76 ± 0.03 a	0.0	5.36 ± 2.1 a	1190 ± 192 a	0.0
B. safensis RF69	1.77 ± 0.1 b	0.17 ± 0.01 b	76.2	4.26 ± 1.4 b	451.0 ± 51 b	62.1
B. amyloliquefaciens RP103	0.34 ± 0.1 c	0.03 ± 0.01 c	95.4	4.16 ± 1.2 c	46.6 ± 9 d	96.1
B. subtilis RP242	0.33 ± 0.1 c	0.02 ± 0.01 c	95.6	4.8 ± 1.2 c	60.7 ± 9 c	94.9

Data are means and standard deviations. Different letters within the same column indicate significant difference ($p \leq 0.05$, Tuckey test). Growth inhibition percentage and OTA production reduction ware calculated by the following formula: [(Control-Treatment)/Control]*100.

lag phase, and therefore, decrease the its capacity to compete and survive, characterizing a possible control method for this fungus (Cavaglieri et al., 2005). The ability to reduce conidial production is also an interesting characteristic to potential biocontrol agents, since the propagation of spores carried by the air is the main dissemination method used by filamentous fungi and its inhibition affects the fungal capacity to invade other substrates.

Ochratoxin A final concentrations were also affected by interaction with the biocontrol agents. All the three *Bacillus* sp. isolates showed to reduce final OTA concentrations in a range between 62 and 96% (Table 1). Accordingly to Choudhary (1992), reduction of toxins levels can be attributed to physical competition for space and nutrition, competition with the fungus for a substrate required for toxin production, induction for a change in the biochemical environment influencing the metabolic pathway available to the toxigenic fungi or degradation of OTA following its formation.

Isolates *B. amyloliquefaciens* RP103 and *B. subtilis* RP242 were the most effective on reducing OTA production. Similar results were observed by Petchkongkaew et al. (2008), which reported *in vitro* reduction of *A. westerdijkiae*'s production of OTA by several *Bacillus* sp. isolates and found a *B. licheniformis* isolate that reduced OTA production by 92%. In addition, Shi et al. (2014) reported the degradation of 97% of OTA levels using a cell-free supernatant from one strain of *B. subtilis* and its ability to inhibit mycelial growth by 33% of another *Aspergillus* section *Circumdati*, *A. ochraceus*, which is a close related to *A. westerdijkiae's* (Visagie et al., 2014). OTA degradation was also reported by Chang et al. (2015), which observed mycotoxin degradation using cells and cell-free metabolites of one strain of *B. amyloliquefaciens*. Our findings and those reported by Petchkongkaew et al. (2008) confirm the potential biotechnological applications of these bacteria on reducing OTA production by *A. westerdijkiae* and its growth on coffee beans.

4 Conclusions

The results obtained in this study present the promising characteristics of *Bacillus* spp. to be used as biocontrol agents to ochratoxigenic *Aspergillus westerdijkiae*. Our findings show that isolates *B. amyloliquefaciens* RP103 and *B. subtilis* RP242 are extremely efficient on inhibiting vegetative growth, the dissemination capacity and OTA production of *A. westerdijkiae* in a coffee based medium as substrate and may be used as an alternative control method aiming the improvement of coffee microbiological and toxicological quality. Further studies must be conducted to evaluate the effect of the bacterial treatment on *A. westerdijkiae* growth and OTA production directly in coffee beans to confirm the commercial usage of the selected biocontrol agents.

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