INHIBITION OF *Fusarium verticillioides* AND *Aspergillus flavus* GROWTH AND MYCOTOXIN PRODUCTION ON MAIZE GRAINS BY *Bacillus* spp.

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ABSTRACT – Filamentous fungi like *Aspergillus flavus* and *Fusarium verticillioides* contaminating maize grains leads to yield losses and quality decrease and may be hazardous to the consuming population due to mycotoxin production. Biological control is a promising alternative control method due to low costs, high efficacy and low toxicity. The present study aimed to evaluate the ability of three *Bacillus* spp. on controlling *A. flavus* and *F. verticillioides* growth and mycotoxin production on maize grain. Maize grains were inoculated with *A. flavus* or *F. verticillioides* and the biocontrol agents. Fungal counts were conducted to evaluate growth and mycotoxin concentration was determined by HPLC and LC-MS/MS. All three *Bacillus* spp. showed to decrease both *A. flavus* and *F. verticillioides* growth and mycotoxin production on maize grains in comparison with the control treatments. The results showed that the biocontrol candidates may be an effective control method aiming fungal and mycotoxin control on maize grains.

KEYWORDS: Aflatoxin B1; Fumonisin B1; Biological control; food mycology; corn.

1. INTRODUCTION

Maize is an important cereal worldwide and grain production is mainly destined for animal and human food (NCGA, 2015). Grains may be contaminated by a diverse number of filamentous fungi both at pre-harvest and during storage. Among several species with the spoiling potential, *Fusarium verticillioides* and *Aspergillus flavus* are the most important ones. The former is a specialized phytopathogen, being responsible for destructive diseases worldwide leading to great yield losses during maize cultivation and grain degradation on poor storage conditions. *Aspergillus flavus* is an opportunistic fungus that can spoil maize grain at pre-harvest on susceptible maize plants and during storage where variations of water activity and temperature lead to fungal proliferation on grain mass (Abbas et al. 2009).

Apart from yield losses and grain degradation, the growth of both fungi may lead to mycotoxin production that characterizes a serious hazard on the safety of grain consumers, both animal and human. *Fusarium verticillioides* is potential producer of fumonisin B1 (FB1), a hepatotoxic, genotoxic and cytotoxic mycotoxin, that has been classified by the International Agency
of Research on Cancer (IARC) as group 2: possibly carcinogenic to humans (IARC, 2002; Escrivá et al. 2015).

Likewise, *A. flavus* may produce aflatoxin B1 (AFB1), a mycotoxin with hepatotoxic, genotoxic and teratogenic characteristics (Binder et al. 2007; IARC, 2012). In addition, IARC classified AFB1 as group 1: carcinogenic to humans and animals (IARC, 2002). Methods of fungal control are extremely necessary to minimize fungal damage and mycotoxin accumulation, avoiding human and animal exposure to AFB1 and FB1 (Chulze, 2010).

Alternative control methods emerged to minimize the impacts caused by synthetic fungicides, aiming a greener agricultural production while containing the phytopathogens damage. Among different methods to prevent fungal colonization, biological control using native microorganisms emerged with great potential due to its usage during both at pre-harvest, through direct application on maize ears, and in storage, by application on the grain mass (Khafanari et al. 2007). Advantages of using living organisms aiming fungal control ranges from low environmental impact to high efficacy on fungal control and the absence of resistance induction to the target pathogens (Chulze et al. 2014).

Bacteria from *Bacillus* genus present promising characteristics to be used as biocontrol agents: resistance to adverse environmental conditions due to its spore forming ability, production of a high variety of antifungal compounds and low nutritional requirements, that facilitates its cultivation and therefore application as commercial biofungicides (Pérez-García et al. 2011).

The aims of this study were to evaluate the ability of three *Bacillus* sp. isolates obtained from maize root system to control *A. flavus* and *F. verticillioides* growth and mycotoxin production on maize grains.

### 2. MATERIALS AND METHODS

#### 2.1 Bacterial and fungal isolates

Three *Bacillus* sp. isolates previously obtained from the maize root system were used: *Bacillus safensis* RF69; *Bacillus amyloliquefaciens* RP103 and *Bacillus subtilis* RP242. One isolate of previously isolated fumonisin B1 producer *F. verticillioides* and aflatoxin B1 producer *A. flavus* were used.

#### 2.2 Inoculation and culture media

Petri plates were filled with 70 g of autoclaved maize grains, centrally sprayed with 1 ml of each *F. verticillioides* or *A. flavus* suspensions (10⁶ conidia ml⁻¹) and *Bacillus* spp. suspensions (10⁹ cells ml⁻¹) grown overnight on 523 broth media (Kado and Heskett, 1970; Bluma and Etcheverry, 2006). Treatment and control plates (sprayed with sterile 523 broth instead of bacterial suspensions) were incubated in triplicates at 25 ºC for 10 days.

#### 2.3 Fungal viable counts

Dilution plating and surface spreading techniques were used for fungal enumeration. Samples of inoculated maize (10 g) were diluted on 90 ml of peptone water (0.01%), shaken of orbital shaker for 30 minutes, diluted serially and aliquots were inoculated on petri plates containing agar DRBC for fungal enumeration (Bluma e Etcheverry, 2006; Pitt e Hocking, 2009). Colonization of maize was assessed as CFU g⁻¹ maize.

#### 2.4 Mycotoxin extraction, clean-up and quantification

Aflatoxin B1 and fumonisin B1 extraction was conducted by the method proposed by Garcia et al. (2012) with a few modifications. Briefly, 10 g of milled inoculated maize samples were extracted with 15 ml of acetonitrile:water (60:40, v.v.) (AFB1) or with 15 ml of methanol:acetonitrile:water
(25:25:50, v/v/v) + 1 g of NaCl (FB1) and shaken on orbital shaker for 20 minutes. Extracts were filtered, mixed with PBS solution and cleaned on the imunoaffinity columns AflaTest and FumoniTest (VICAM, Watertown, MA, USA), respectively.

The method of Oliveira et al. (2015) was used for fumonisin B1 analysis by liquid chromatography-mass spectrometry (LC-MS/MS). The recovery obtained by this method was 98.8% for fumonisin B1 (Oliveira et al. 2015).

For AFB1 analysis, samples were resuspended on 0.6 ml ammonium acetate (10mM) and injected on HPLC/MS. The liquid chromatography column used was a Zorbax Eclipse C18, 4.6 x 50 mm, 1.8 μm pore (Agilent Technologies Inc., USA) on the following parameters: Flow rate of 0.6 mL/min; column temperature of 40 ºC, injection volume of 5 μm. Water:ammonium acetate (10mM) was used as eluent A and methanol as eluent B. The MS source dependent parameters were: source temperature 350 ºC, dry gas 10 L min⁻¹ and the spray voltage was set to 1000 V. Detection (LOD) and quantification (LOQ) limits were 0.06 and 0.2 ng g⁻¹ and recovery rate was 98.2%.

2.5 Statistical analyses

One-way ANOVA was applied to data analyzes, using Statistica 10.0 (StatSoft, Inc. (2011) software for Windows. Tuckey test was conducted for treatments mean comparison using p<0.05 significance.

3. RESULTS AND DISCUSSION

The results of A. flavus and F. verticillioides counts on maize grains after the bacterial treatments are showed on Table 1. All Bacillus spp. isolates were able to reduce A. flavus growth on a range between 19 and 48% and F. verticillioides growth on a range between 20 and 43%. These results suggest a possible application of the selected biocontrol agents directly on maize ears in the field or in stored systems. Although all isolates presented significant reduction of fungal counts, the isolate B. amyloliquefaciens RP103 showed to be more effective (p<0.05) on controlling both A. flavus and F. verticillioides on maize grain in comparison with the isolates B. safensis RF69 and B. subtilis RP242. Similar results were observed previously by Bluma and Etcheverry (2006) and Etcheverry et al. (2009), which reported significand A. flavus inhibition by several Bacillus spp. isolates and by Cavagliieri et al. (2005) and Pereira et al. (2010) which found high inhibition of F. verticillioides by B. amyloliquefaciens and other Bacillus species.

Table 1 - Effect of Bacillus spp. treatments of maize grains on A. flavus and F. verticillioides counts

<table>
<thead>
<tr>
<th></th>
<th>Fungal counts (log CFU g⁻¹ maize)</th>
<th>Fungal growth inhibition (%)</th>
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<tbody>
<tr>
<td></td>
<td>A. flavus</td>
<td>F. verticillioides</td>
</tr>
<tr>
<td>Control</td>
<td>8.7 ± 2.08 a</td>
<td>7.8 ± 2.07 a</td>
</tr>
<tr>
<td>B. safensis RF69</td>
<td>7.0 ± 2.64 b</td>
<td>6.9 ± 2.64 b</td>
</tr>
<tr>
<td>B.amyloliquefaciens RP103</td>
<td>4.6 ± 1.91 d</td>
<td>5.0 ± 1.91 d</td>
</tr>
<tr>
<td>B. subtilis RP242</td>
<td>5.7 ± 1.96 c</td>
<td>6.4 ± 1.93 c</td>
</tr>
</tbody>
</table>

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

The AFB1 and FB1 production and accumulation on maize grains treated with Bacillus spp. are showed on Table 2. All Bacillus sp. isolates were capable of reducing AFB1 and FB1 production and accumulation after the treatment period, in a range between 55 - 95% and 45 - 82% for AFB1 and
FB1, respectively. Likewise the fungal counts observed, the isolate B. amyloliquefaciens RP103 showed significantly higher reduction (p<0.05) of AFB1 final concentrations when compared with the other two Bacillus isolates, being capable of near complete mycotoxin reduction (~94% reduction) after the 10 days treatment. Regarding FB1 production, both B. amyloliquefaciens RP103 and B. subtilis RP242 presented higher reduction rates when compared with the isolate B. safensis RF69. Our results are in agreement with those obtained by Bluma and Etcheverry (2006), which reported reduction of final AFB1 concentrations of maize treated with different strains of Bacillus amyloliquefaciens and with those obtained by Pereira et al. (2010) that found reduction of FB1 on maize ears after application of another strain of B. amyloliquefaciens.

Table 2 - Effect of Bacillus spp. treatments of maize grains on aflatoxin B1 production by A. flavus and fumonisin B1 production by F. verticillioides

<table>
<thead>
<tr>
<th></th>
<th>Mycotoxin production (mg kg⁻¹)</th>
<th>Mycotoxin production inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aflatoxin B1</td>
<td>Fumonisin B1</td>
</tr>
<tr>
<td>Control</td>
<td>0.8 ± 0.1 a</td>
<td>2.9 ± 0.3 a</td>
</tr>
<tr>
<td>B. safensis RF69</td>
<td>0.3 ± 0.08 b</td>
<td>1.5 ± 0.7 b</td>
</tr>
<tr>
<td>B. amyloliquefaciens RP103</td>
<td>0.04 ± 0.02 d</td>
<td>0.5 ± 0.1 c</td>
</tr>
<tr>
<td>B. subtilis RP242</td>
<td>0.2 ± 0.03 c</td>
<td>0.5 ± 0.8 c</td>
</tr>
</tbody>
</table>

Data are means and standard deviations. Different letters within the same column indicate significant difference (p≤0.05, Tuckey test). Inhibition percentage was calculated by the following formula: [(Control-Treatment)/Control] *100

4. CONCLUSIONS

In summary, the results obtained in this study present the promising characteristics of Bacillus spp. to be used as biocontrol agents to Aspergillus flavus and Fusarium verticillioides on maize agroecological systems. All Bacillus isolates were capable of reducing growth and mycotoxin production of A. flavus and F. verticillioides on maize grain on the tested conditions, indicating an improvement of both microbiological and toxicological quality of the maize. Further studies need to be conducted to evaluate the effect of the selected biocontrol agents on F. verticillioides and A. flavus colonization and mycotoxin accumulation on maize plants on a field scale to confirm the potential usage of these biocontrol agents.

5. ACKNOWLEDGEMENTS

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6. REFERENCES


