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Managing and monitoring of *Aspergillus flavus* in corn using bioplastic-based formulations

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ABSTRACT

In this study, we evaluated the feasibility of bioplastic-based formulations for delivering a non-aflatoxigenic strain of *Aspergillus flavus* and for monitoring Aspergilli with the final objective of controlling aflatoxin contamination in corn. Field application of inoculated bioplastic granules showed a rapid shift in the composition of soil *A. flavus* population, with a significant decrease in relative abundance of indigenous aflatoxigenic isolates. Application of bioplastic granules at 30 kg ha^{-1} was more efficient in replacing aflatoxigenic isolates than a 15 kg ha^{-1} dosage. In the test plots evaluated, aflatoxin contamination levels at corn maturity were 4.4 and 28.9 ng g^{-1} for the 2009 and 2010 field seasons, respectively. However, the biocontrol formulation was effective in reducing aflatoxin contamination in both years. More precisely, soil application of 15 and 30 kg ha^{-1} of bioplastic granules reduced aflatoxin contamination by 59 and 86% in 2009, and 80 and 92% in 2010, respectively.

1. Introduction

Aflatoxins refer to a group of mycotoxins mainly produced by the filamentous fungi *Aspergillus flavus* and *Aspergillus parasiticus* (Yu et al., 2008). Since the discovery of aflatoxins in the early 1960s, numerous studies have focused on the ecology of *A. flavus* in the agro-ecosystem (Wicklow et al., 1998; Abbas et al., 2008; Accinelli et al., 2008). Some of the crops most susceptible to aflatoxin contamination include peanuts, cotton and corn (Scheidegger and Payne, 2003). The severity of aflatoxin contamination is largely determined by the environment, with pre-harvest contamination being favored under drought and temperature stress (Payne, 1992, 1998; McGee et al., 1996; Abbas et al., 2007). *A. flavus* is a ubiquitous fungus readily isolated from diverse environments, with soil and plant tissues or residues being the natural habitat of this fungus (Geiser et al., 2000; Horn, 2003; Abbas et al., 2009). Since soil serves as a reservoir for primary inoculum for the infection of susceptible crops, most studies have focused on the occurrence and quantification of Aspergilli propagules in soil (Horn and Dorner, 1998; Zablotowicz et al., 2007; Accinelli et al., 2008). During the last two decades, several pre-harvest strategies for reducing aflatoxin contamination have been proposed (Abbas et al., 2009). An innovative approach is a biocontrol strategy consisting in the use of non-aflatoxigenic isolates of *A. flavus* to competitively exclude indigenous aflatoxigenic Aspergilli (Brown et al., 1991; Abbas et al., 2006). A 4-year study conducted in the Mississippi Delta demonstrated that soil application of wheat grains inoculated with the non-aflatoxigenic strain *A. flavus* NRRL 30797 was successful in reducing aflatoxin contamination in corn (Abbas et al., 2006). Subsequent laboratory studies showed that grains can be efficiently replaced by granules made of the bioplastic Mater-Bi® (Accinelli et al., 2009). In addition to having a favorable environmental profile, the starch-based bioplastic matrix promotes vigorous growth and sporulation of the fungus, thus facilitating soil colonization. This property can also have other practical implications. For instance, it can be expected that the starch-based bioplastic would serve as a baiting material for isolation of Aspergilli. In this two-year study, we investigated the feasibility of bioplastic granules entrapping propagules of the non-aflatoxigenic isolate *A. flavus* NRRL 30797 for controlling aflatoxin contamination in corn. We also monitored the soil Aspergilli population and corn kernel infestation using conventional protocols and a novel bioplastic-based formulation specifically developed for facilitating recovery of Aspergilli from environmental samples.

2. Materials and methods

2.1. Preparation of inoculated bioplastic granules

The non-aflatoxigenic strain *A. flavus* NRRL 30797, isolated in the Mississippi Delta in 2001, was selected for this study. Detailed

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properties of strain NRRL 30797 are described elsewhere (Abbas et al., 2006; Abbas et al., 2011). The fungus was grown and maintained on acidified potato dextrose agar (PDA). After incubation for two weeks at 37 °C, spores were removed by gently scraping PDA plates and suspended in aqueous 0.2% Tween 20. Density of spore suspensions was determined using a hemocytometer and adjusted as necessary. Spores were then entrapped into spherical granules (diameter 3 mm) made of the bioplastic Mater-Bi®(MB) type PE01S (Novamont S.p.A., Novara, Italy), following the procedure described in Accinelli et al. (2009). Briefly, bioplastic granules were equilibrated at room temperature with a concentrated spore suspension (1:1.25 w/v) with rotary shaking at 300 rpm. After 4-h shaking, spore suspensions were forced through granules by pressure (60 kPa) and granules were dried at 40 °C. Inoculated granules were stored at room temperature for no longer that one week. Potency of the final product (number of viable spores entrapped in granules) was determined by plate count. Granules were transferred to centrifuge tubes (3 granules/tube) containing 10 ml of phosphate buffer saline (PBS) and glass beads. After vortexing for 3 min, tubes were shaken at 300 rpm for 1 h. Suspensions were serially diluted in PBS and 100 μl aliquots were plated onto modified dichloronitroaniline rose bengal agar (MDRBA; Abbas et al., 2004b). Colonies were enumerated after 7–10 days of incubation at 37 °C.

2.2. Site description and experimental design

The study was carried out during two consecutive years (2009–2010) at the experimental farm of the University of Bologna (Bologna, Italy). For each year, experiments were conducted in single adjacent 1.5-ha corn fields. Physico-chemical properties of soil of the two selected fields have been reported elsewhere (Accinelli et al., 2002). Experiments were conducted using a completely randomized block design with three replicates. Each experimental unit consisted of a 600-m^2 area (30 m × 20 m) surrounded by a 10-m wide buffer zone. Experimental treatments were the following: inoculated bioplastic granules at the rate of 15 and 30 kg ha^{-1}, and an untreated control. A conventional corn hybrid (Pioneer Hi-Bred PR31K18) was planted on 16 April 2009 and 12 April 2010. Fields were managed according to ordinary practices of the region. Bioplastic granules were uniformly spread by hand on the ground surface of each plot at corn growth stage V4 (Ritchie and Hanway, 1982). Corn was harvested on 25 August 2009 and 27 August 2010. A total of 60 ears were randomly collected from each plot, shelled and dried at 50 °C for 72 h and ground (<1 mm) for chemical analysis.

2.3. A. flavus population

Size (propagule density) of the *A. flavus* soil population and relative abundance of non-aflatoxigenic isolates were measured over the two corn-growing seasons. At each sampling time, three surface (0–10 cm) soil samples were collected from each plot. Samples were sieved through a 4-mm sieve and stored at 4 °C until processed. Soil moisture was determined gravimetrically. Enumeration of *A. flavus* propagules was performed following the procedure described in Abbas et al. (2004b) with minor modifications. Briefly, 10 g of soil were suspended in a 90-ml water agar solution (0.2%), vortexed for 3 min, and shaken for 1 h at 300 rpm. Suspensions were used for preparing ten-fold serial dilutions in PBS, with 100-μl aliquots plated onto MDRBA and incubated at 37 °C for 7–10 days. Ten colonies were randomly selected and subculture on PDA at 28 °C. After seven days of incubation in the dark, aflatoxin-producing isolates were identified as colonies that displayed blue fluorescence during exposure to UV light (365 nm). At crop maturity, in addition to ears collected for chemical analysis, 10 more corn ears were collected as describe above and used for microbiological analysis. After drying at 50 °C for 72 h, kernels were removed from ears, and a randomly selected number of kernels were surface sterilized and plated onto MDRB agar. After incubation for 7–10 days at 37 °C, kernels showing *A. flavus* infection were recorded and a selected number of isolates were assessed for their potential to produce aflatoxins after UV-exposure (Abbas et al., 2004b).

2.4. Recovering Aspergilli using a bioplastic-based bait formulation

In addition to the cultivation-based method a bioplastic-based baiting system was developed. For baiting Aspergilli, MB bioplastic was shaped as rods (diameter 2 mm, length 40 mm; MB type ZF03U/A) and granules (diameter 3 mm; MB type PE01S). Autoclaved rods and granules were infiltrated with sterile MDRB by suspending them in the broth for 3 h at 300 rpm and 40 °C, and drying under a laminar flow hood for 2 h. Rods and granules were then stored at 4 °C until use. The soil incubation study was carried out using soil collected at corn maturity from the field selected in 2010. Triplicate surface samples (0–10 cm) were collected from each plot, sieved at 4 mm and 25 g (air-dried weight equivalents) were weighed in 50-ml sterilized screw-top tubes and moisture adjusted to the gravimetric content at −33 kPa. Rods (4 rods per sample) were then inserted into the soil mass and samples incubated in the dark at 28 °C. After 14 days of incubation, rods were aseptically removed and processed to quantify Aspergilli DNA by quantitative PCR (qPCR). Rods were cut in four equal parts and processed as described in Accinelli et al. (2009). Briefly, rod parts were dried at 40 °C for 2 h under a laminar flow hood, transferred to 2-ml centrifuge tubes, vortexed for 5 min to remove adhering soil particles and air-flushed by high-pressure air. Each dried rod fragment was transferred to a 2-ml microcentrifuge tube containing 500 μl of CTAB buffer and glass beads. After vortexing for 2 min, tubes were incubated at 65 °C for 15 min, and an equivalent volume of chloroform:isoamyl alcohol (24:1, v:v) was added to tubes. Tubes were gently shaken and centrifuged at 10,000*g* for 5 min before the addition of 2/3 volume of isopropanol/7.5 M ammonium acetate to precipitate the DNA. The pellet was rinsed with 70% ethanol, air dried and resuspended in 100 μl of TE buffer.

Ten ears per plot were randomly collected at maturity from the same field and used for baiting Aspergilli from kernels. Twenty grams of dried kernels were weighed in 50-ml sterilized screw-top tubes containing 10 MRB-infiltrated bioplastic granules. After vortexing the tubes for 5 s, tubes were incubated at 28 °C in the dark for 10 days, granules were aseptically removed and used for DNA isolation to conduct qPCR analysis as described above. Remaining granules were used for assessing the potential of baited isolates to produce aflatoxin. A single granule was transferred to a test tube containing 2 ml of yeast extract sucrose broth and incubated at 30 °C. After incubation for seven days in the dark without shaking, cultures were extracted with chloroform. Chloroform was then evaporated to dryness under vacuum and residues redissolved in methanol/H_2O (70:30, v:v). Dry weight of mycelium was determined after drying the mycelia mats for 48 h at 70 °C. A selected number of isolates (42) recovered from the surface of bioplastic granules were transferred to PDA plates and used for DNA sequencing. The experiment was repeated using 25 samples of corn kernel provided by two private laboratories (AGER Bologna and Caip Bologna-Modena). Aflatoxin concentration of these samples was determined by using the same HPLC method described below.

2.5. DNA analysis

Amplification was carried out in a total volume of 25 μl containing 2 μl of DNA, 12.5 μl of 2× TaqMan Universal PCR Master Mix