Crop Protection 63 (2014) 9–14

Contents lists available at ScienceDirect

Crop Protection

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

Aflatoxin contamination of corn under different agro-environmental conditions and biocontrol applications

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ARTICLE INFO

Article history:
Received 20 January 2014
Received in revised form
25 April 2014
Accepted 26 April 2014
Available online 22 May 2014

Keywords:
Aflatoxin
Aspergillus flavus
Bioplastic
Biopesticides
Zea mays
Bt corn

ABSTRACT

Biological control of the fungus *Aspergillus flavus* has been shown to be effective in reducing aflatoxin contamination in corn. This study compared field application of a bioplastic-based formulation for delivering atoxigenic *A. flavus* isolates in Northern Italy and the Mississippi Delta.

Due to an extremely hot and dry summer at the Italy site in 2012, aflatoxin contamination was approximately seven times higher than in 2011. In 2011, and 2012, application of bioplastic granules inoculated with the atoxigenic isolate *A. flavus* NRRL 30797 at 15 and 30 kg ha^{-1} resulted in a reduction of aflatoxin contamination by 67.2 ± 4.1% and 94.8 ± 5.3%, respectively. The higher application rate was also effective when soil abundance of *A. flavus* was artificially increased by applying contaminated corn residues. At the Mississippi site, summer 2012 was also hot and dry, with high levels of aflatoxin contamination. In fields planted with non-Bt or Bt hybrids, application of biocontrol granules inoculated with *A. flavus* NRRL 30797 or NRRL 21882 at 30 kg ha^{-1} reduced aflatoxin contamination to up to 89.6%. Field experiments on two continents showed that bioplastic-based *A. flavus* formulations markedly reduced aflatoxin contamination under different agro-environmental conditions and infestation intensities.

1. Introduction

The need for agriculture to give increased yields and be more sustainable has prompted research and development to focus on identifying new and practical pest control methods that do not depend on chemical pesticides. New approaches have included introducing chemical pesticides with a more benign environmental profile and increased selectivity, the adoption of pest-resistant or pest-tolerant crops and the use of novel and effective microbial biocontrol agents. Among the various methods for aflatoxin control, the application of augmentative biological control for reducing aflatoxin contamination in peanuts, cotton and corn is certainly one example of a successful application of the strategy (Amaike and Keller, 2011). Aflatoxins are toxic, hepatocarcinogenic secondary metabolites produced mainly by the fungus *Aspergillus flavus* and closely related species (Payne and Brown, 1998). *A. flavus* is ubiquitous in the environment with soil and crop residues being its natural habitat (Abbas et al., 2008a, 2008b). In the soil, the fungus lives as a saprophyte participating in the cycle of soil organic matter. Conidia produced by *A. flavus* in soil are dispersed by wind, insects and other vectors, enabling them to inoculate new host plants. In corn the fungus colonizes silk tissues, then grows down the silks to the kernels where it can infect developing kernels and go on to produce aflatoxins (Scheidegger and Payne, 2003). Infection and colonization of tissues are facilitated when plants are stressed, for example by high temperature and drought (Abbas et al., 2008b).

Aflatoxin contamination of food and feed is a serious health concern worldwide and consequently it is regulated in many countries (Wu and Khlangwiset, 2010). Since direct and indirect economic losses due to aflatoxin contamination may severely impact profitability of corn producers, strategies for controlling pre-harvest contamination by aflatoxins are of great practical interest (Abbas et al., 2008b; Cleveland et al., 2003) and among those strategies is augmentative biocontrol (Cotty, 1994; Ehrlich and Cotty, 2004; Abbas et al., 2006). More specifically, this biocontrol technique is based on the application of a large number of

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http://dx.doi.org/10.1016/j.cropro.2014.04.021
0261-2194/

propagules, mainly spores, of selected nontoxigenic *A. flavus* strains to the soil of developing crops (Dorner, 2004; Abbas et al., 2008b). An important prerequisite for any *A. flavus* biocontrol strain is that it should be nontoxigenic, easily produced and applied, and capable of competing effectively with indigenous isolates Dorner (2004). Typically, biocontrol isolates are applied to agricultural fields as inoculated or spore-coated grain seeds. A biocontrol formulation consisting of spore-coated barley seeds has been commercialized by Syngenta Crop Protection with the name Afla-Guard®. However, more recently, the possibility to replace grain seeds with bioplastic-based granules manufactured from destructured starch has been explored (Accinelli et al., 2009, 2011). Field experiments conducted in Northern Italy have demonstrated that bioplastic formulations are effective in reducing aflatoxin contamination in corn (Accinelli et al., 2012). This study also showed that application of the biocontrol agent to the same soil on consecutive years resulted in a greater relative reduction in aflatoxin contamination of corn kernels and toxigenic *A. flavus* population in soil in the subsequent year.

The main objectives of the present study were to investigate aflatoxin contamination of corn under different agro-environmental conditions, in the presence of different levels of *A. flavus* infestation, and using different biocontrol application techniques.

2. Materials and methods

2.1. Fungal isolates, bioplastic granules and corn residues

These studies were conducted using the following two biocontrol isolates: *A. flavus* NRRL 30797 (Abbas et al., 2006, 2011a, 2011b) and *A. flavus* NRRL 21882 (Dorner, 2009). Fungi were grown on potato dextrose agar (PDA) at 28 °C for 7–10 days, then ten 5-mm agar plugs were transferred to autoclavable plastic bags containing 1 kg of pre-wetted and sterilized granules (diameter 3 mm) made of the starch-based bioplastic Mater-Bi® (MB) type PE01S (Novamont S.p.A., Novara, Italy). The bags were capped with cotton plugs, fixed to steel rings and incubated in vertical position for 10 days at 28 °C. Inoculated granules were then dried at 45 °C in a ventilated oven for two days and stored at room temperature for ≤ one week before use.

Corn residues (CR) were collected in September, 2011, from a commercial corn field close to where the biocontrol experiments were conducted, dried at 45 °C and cut into 4 mm × 4 mm pieces. CR were autoclaved at 120 °C for 30 min on three successive days, then inoculated by immersing in a spore suspension prepared by mixing spores from 10 *A. flavus* toxigenic and 10 atoxigenic strains. The 20 *A. flavus* isolates were indigenous strains isolated in a previous field study (Accinelli et al., 2012). Fungal isolates were grown on PDA for 7 days at 28 °C and spores were collected by flooding the plates with 5 mL of 0.2% Tween-20 in sterile distilled water. After evaluating spore density, spores of the 20 isolates were dispersed in equal amounts in water at 1×10^7 spore/ml. The inoculated CR was dried in a laminar flow hood and stored at 4 °C for one week before use.

2.2. Experimental sites and treatments

Experiments were conducted in commercial corn fields located near Bologna, Italy, (IT) and Stoneville, Mississippi, USA, (MS). At the IT site, experiments were carried out during two consecutive years (2011–2012) in adjacent 1.5-ha corn fields. Each plot consisted of a 600-m^2 area (30 m × 20 m) surrounded by a 10-m wide buffer zone. Bioplastic granules inoculated with *A. flavus* NRRL 30797 were applied at 15 and 30 kg ha^{-1} in both years. In 2012, CR was also uniformly applied to the soil surface at 30 kg ha^{-1} two days before biocontrol treatment. In 2012 at the MS site two adjacent 0.5-ha corn fields were divided into 150 m^2 (20 m × 7.5 m) experimental units separated by 3-m wide buffer zones of corn crop. The following corn hybrids were planted: glyphosate- and gluphosinate-resistant hybrid 31G97 (relative maturity 117 days; Pioneer Hi-Breed, Des Moines, Iowa) and the Bt-protected YieldGard VT Triple® DKC63-42 hybrid (relative maturity 113 days; DeKalb Genetics, DeKalb, Illinois). In both fields, granules inoculated with *A. flavus* NRRL 30797 or NRRL 81882 were applied at 30 kg ha^{-1}. At the IT and MS locations, granules were uniformly spread on the ground surface at the corn growth stage V4 (Ritchie and Hanway, 1982) using a hand-held spreader (The Scotts Company LCC, Marysville. Ohio).

Fields were managed according to conventional practices of the two regions. At the beginning of September, the two middle rows from each plot were harvested, and the corn dried at 50 °C for 72 h to <14% moisture content, then samples for chemical and microbiological analysis were ground to <1 mm using a Romer mill. Six surface (0–10 cm) soil samples were collected at random from each plot, sieved through a 4-mm sieve and stored at 4 °C until processed for microbiological analysis.

2.3. Aspergillus flavus in soil and corn kernels

Enumeration of *A. flavus* propagules of soil and ground corn kernels was performed by published procedures (Abbas et al., 2004b; Accinelli et al., 2012). Briefly, 10 g of soil or ground corn were suspended in 90-ml of aqueous agar solution (0.2%) containing glass beads, vortexed for 3 min, and shaken for 1 h at 300 rpm. Suspensions were used to prepare 10-fold serial dilutions in PBS, from which 100-μl aliquots were plated on modified dichloronitroaniline rose Bengal (MDRB) agar and incubated at 37 °C for 7–10 days. Ten colonies were randomly selected and subcultured on PDA at 28 °C for 7 days in the dark. Aflatoxin-producing isolates were identified as colonies displaying blue fluorescence on exposure to UV light (365 nm).

In addition to the cultivation-based method, the potential of soil and corn kernel isolates to produce aflatoxin was evaluated using a rapid method based on the use of bioplastic-based baits (Accinelli et al., 2012). Briefly, four rod-shaped bioplastic baits were transferred to each 25 g sample of 4-mm sieved soil and incubated at 28 °C for 10 days. Single baits were transferred to test tubes containing 2 ml of yeast extract sucrose broth and incubated without shaking for 7 days in the dark at 30 °C, then extracted with chloroform. Chloroform extracts were evaporated to dryness *in vacuo* and the residues dissolved in methanol/H_2O (70:30, v:v) and the aflatoxin concentration determined by HPLC as described below. Dry weight of mycelia mats was determined after air drying 48 h at 70 °C.

2.4. Aflatoxin analysis

Aflatoxin levels in corn kernel samples were determined as described elsewhere (Abbas et al., 2006, 2008a). Briefly, 20 g samples of ground corn were extracted with 100 mL of methanol/water (70:30) at room temperature for 3–5 min with high speed (150 rpm) stirring. The crude extract was clarified by filtration using Whatman grade 1 filter paper and further clarified by centrifugation at 5,000 × g for 10 min, if required, then stored at −20 °C until analyzed. Samples were warmed to room temperature, 5 mL-aliquots of clear extract taken for clean-up on a minicolumn packed with aluminum oxide (Alltech Co., Deerfield, IL), and a 20 μL of the eluate injected on an HPLC system equipped with a Nova-Pak C18 column (150 × 3.9 mm, 4 μm) and a multi-wavelength fluorescence