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Biological control of aflatoxin production in corn using non-aflatoxigenic *Aspergillus flavus* administered as a bioplastic-based seed coating

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ABSTRACT

Biocontrol techniques for reducing aflatoxin contamination in corn kernels usually involve massive application of non-aflatoxigenic *A. flavus* to soil. In this study, applying biocontrol agents directly to corn by incorporation into a seed coating was explored. Seeds were film-coated with a starch-based bioplastic formulation containing two conventional pesticides (insecticide: imidacloprid; fungicide: metalaxyl-M) and spores of non-aflatoxigenic *A. flavus* NRRL 30797. Application of the bioplastic seed-coating with or without additives did not affect seed germination or seedling growth. The coating remained adherent to seed surfaces, reducing seed dust release. Incorporating biocontrol *A. flavus* into the bioplastic seed coating resulted in a decreased percentage of aflatoxin-producers in recoverable field soil isolates and significantly lower aflatoxin contamination of harvested corn kernels relative to seed coating with pesticides alone in the following field locations: (i) Northern Italy in 2016, where biocontrol seed coating reduced aflatoxin contamination from 7.1 to 2.1 ng g^{-1}; (ii) Mississippi Delta in a low aflatoxin contamination field, where biocontrol seed coating reduced aflatoxin contamination from 5.8 to 3.1 ng g^{-1} in 2015 and from 33.4 to 8.2 ng g^{-1} in 2016; and (iii) Mississippi Delta in a high aflatoxin contamination field, where biocontrol seed coating reduced aflatoxin contamination from 74.4 to 15.1 ng g^{-1} (79.7%) in 2015 and from 95.0 to 16.0 ng g^{-1} (85.2%) in 2016. These results suggest that seed coating may be a useful approach to deliver biocontrol agents for reducing aflatoxin contamination in corn.

1. Introduction

Corn (*Zea mays* L.) is one of the most important cereal crops in the human diet and animal feed worldwide (Wu and Guclu, 2013). In the last decades, advancements in corn genetics, breeding technologies, and agronomic techniques have resulted in a tremendous increase in corn yield (Chavas et al., 2014). Because of high productivity and economic values of modern seed varieties, a rapid, uniform and complete seedling establishment is a key factor for profitable corn production.

As for other agronomic species, seeds of corn hybrids are routinely treated with fungicides and/or insecticides for reducing or suppressing infestation of soil-borne pathogens and damage by insects on germinating seeds and growing seedlings (Taylor et al., 2001; Nuyttens et al., 2013; Pedrini et al., 2017). Application of active ingredients directly to the seeds also reduces the load of pesticides released in the environment compared to broadcast or in-furrow pesticide treatments (Elbert et al., 2008). Except for irregularly shaped seeds or seeds having very small sizes, seeds of most crop species are currently film-coated using basic rotating drum machines (Taylor et al., 1998: TeKrony, 2006; Accinelli et al., 2016a). More specifically, film-coating is achieved by treating seeds with an aqueous suspension consisting of a polymer or a mixture of polymers, plasticizers, sticking agents and pesticide active ingredients. The process results in the formation of a thin layer covering the seeds. Shape and seed size are not altered, however their flowability is improved and pesticide dust-off is reduced by the presence of sticking agents (Taylor et al., 1998; Accinelli et al., 2016a). In the last decade, the need for novel pest control tools with a more favorable environmental profile has stimulated research on effective microbial biocontrol agents and formulations to replace or to reduce the use of synthetic pesticides for field broadcast applications and seed treatment (Mancini and Romanazzi, 2014; Accinelli et al., 2016a, 2016b).

Among the different film-coating technologies, a recent study has introduced the use of a liquid starch-based bioplastic for film-coating seeds of agronomic species, combining propagules of a *Trichoderma* biocontrol isolate with synthetic pesticides. The study showed that, in addition to remaining adherent to the seed surface, the bioplastic coat

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did not affect germination. Most importantly, being a starch-based material, the bioplastic film promoted initial growth of this starch-utilizing fungus. Although *Trichoderma*-based formulations are currently used in a variety of biocontrol programs, applications of this fungus have not been successful in the biocontrol of aflatoxin contamination in vulnerable crops, such as corn (Harman, 2011).

Aflatoxins are a group of carcinogenic mycotoxins produced by the fungus *Aspergillus flavus* and related species (Payne and Brown, 1998; Khlangwiset and Wu, 2010). The fungus can infect a variety of crops, especially oilseed crops, including corn, peanuts and cotton, causing significant direct and indirect economic losses in many agricultural areas worldwide (Wu and Khlangwiset, 2010; Amaike and Keller, 2011). Scientific and technical studies have demonstrated that field applications of competitive non-aflatoxigenic strains of *A. flavus* were effective in reducing aflatoxin contamination in corn under different climate and agronomic conditions (Dorner, 2004; Abbas et al., 2006; Accinelli et al., 2012). Biocontrol isolates of *A. flavus* are commonly applied to field soil using inoculated grains (i.e., deactivated wheat, barley or rice seeds). After application to the soil surface using a common granular fertilizer spreader, these starch-rich grains rapidly absorb moisture, creating conditions for the fungus to resume growth and sporulation. More recently, the possibility of replacing inoculated cereal grains with solid or liquid bioplastic formulations has been proposed (Accinelli et al., 2009, 2016b). These starch-bioplastic formulations are prepared by entrapping spores directly into the solid bioplastic matrix or by suspending the spores in the sprayable bioplastic formulation. In addition to being more flexible in use, the liquid bioplastic formulation has the advantage that the amount of spores to be applied per hectare would be significantly lower than with the soil-applied granular formulation (Accinelli et al., 2016b).

This study evaluated the feasibility of reducing aflatoxin contamination in corn by applying a biocontrol *A. flavus* isolate directly at planting, by means of a novel seed treatment technology. More specifically, the proposed approach consisted of a film-coating formulation combining spores of the biocontrol fungus and two synthetic pesticides commonly used for treating corn seeds. The three components were kept adherent to the seed surface by a starch-based bioplastic, which was also expected to provide a nutrient source for the initial growth of the fungus. In addition to aflatoxin contamination, effects of the bioplastic coat on seed germination, seedling growth, microbiological parameters and seed dust release were evaluated.

2. Materials and methods

2.1. Film-coating components and procedures

Seeds of a commercial corn hybrid (cultivar Yellow Trucker's Favorite, The Wax Company, Amory, MS) were film-coated using a modified rotating drum machine (Kobalt 4-cu ft 0.5-HP, LF, LCC, Mount Mourne, NC). The modification consisted of three 15-cm-wide plastic deflectors, which were mounted at the base of the rotating drum to ensuring a uniform distribution of the coating slurry. Seed treatment was achieved by applying a coating slurry (15 mL kg^{-1} seed) containing 1% (v/w) of a modified grade of the starch-based liquid bioplastic (BL) as described by Accinelli et al. (2016a). This bioplastic grade was obtained by adding chitin (0.5%; w/w) and glycerol (0.2%; w/w) to a destructured starch. The bioplastic coating agent was then combined with the insecticide imidacloprid (Gaucho 600; 600 g imidacloprid per liter; Bayer Crop Science AG, Monheim, Germany), the fungicide metalaxyl-M (Apron XL, 369 g metalaxyl-M per liter; Syngenta Crop Protection AG, Basel, Switzerland) and spores of the non-toxigenic biocontrol isolate *A. flavus* NRRL 30797 (Abbas et al., 2011). Both pesticides were applied at the rate of 1.2 mL kg^{-1} of seeds. Spores of the *A. flavus* biocontrol strain were obtained following the procedure described in Accinelli et al. (2012). Briefly, the fungus was grown on acidified potato dextrose agar (PDA) for 10 days at 28 °C and then the plates were scraped and the spores suspended in a sterilized 0.2% Tween 20 aqueous solution. Spores were counted on a hematocytometer and the concentration of the suspension adjusted to approximately 10^3 spores mL^{-1}. Five milliliters of this spore suspension were then added to 10 mL of the coating slurry containing the two pesticides and the bioplastic. The same seed treatment procedure was used to evaluate the effect of single and combined components (e.g., bioplastic, pesticides, and spores) on seed germination, seedling growth and seed dust release.

2.2. Seed germination, seedling growth and measurement of seed dust release

Seed germination and seedling growth were evaluated as described in Accinelli et al. (2016a). Briefly, for each treatment, 100 seeds of the corn hybrid were randomly selected and placed between rolled moist paper towels (10 seeds per roll) and incubated in a germination chamber at 20 °C (80% relative humidity) with 12 h of light per day. After 14 days of incubation, the number of normal seedlings was recorded.

Seeds from the same batches were then planted in pots and placed in a growth chamber for evaluating seedling growth. Plastic pots (18-cm diameter; 25-cm high) were filled with 350 g of 4-mm sieved topsoil and moisture adjusted to field capacity. Pots were planted with 2 seeds to a depth of 3 cm and maintained in a growth chamber for 21 days under supplemental light for a 12-hr period and day and night temperatures of 25 °C and 15 °C (80% relative humidity), respectively. Plants were watered daily and plant height recorded at 2-day intervals. After a period of 21 days, plants were carefully removed and roots washed under running tap water to remove adhering soil. Shoots and roots were dried and their length was recorded. A total of 60 seedlings for each treatment were measured.

The effect of bioplastic on the amount of dust released from treated seeds was evaluated by the Heubach dust abrasion test, as described in Accinelli et al. (2016a). Briefly, for each treatment, five replicates of 100 g of seeds were weighed and kept for 48 h at 20 °C and 50% relative humidity. A pre-weighed glass fiber filter disc (GF 92; Whatman Inc., Newton, MA) and a sample of 500 g of seeds were transferred to the rotating drum of the Heubach dustmeter (Heubach GmbH, Langelsheim, Germany) that operated for 30 min at 30 rpm with an airflow rate of 20 L min^{-1}. After rotation, the filter was removed and weighed and the rotating drum and all the machine parts were carefully washed with ethanol. Collected dust from treated seeds was expressed as g of dust 100 kg^{-1} seeds.

2.3. Field experiments and aflatoxin analysis

Seeds coated with bioplastic, spores and the two pesticides were planted in mid-April in two commercial fields, which were planted with corn in the previous 4 years. The two fields were located in different areas of Washington County, Mississippi, USA (MS), one with consistently low aflatoxin contamination of corn (MS #1) and one with consistently high aflatoxin contamination of corn (MS #2). The two 8-ha fields were divided into six rectangular blocks (110 m × 120 m) and experimental treatments were arranged according to a completely randomized block design. Each block was surrounded by a 3-m wide buffer area. The same experimental scheme was adopted in a commercial corn field located in Northern Italy (ITA) near Bologna. In all areas, fields were managed according to conventional practices of the 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 were ground to < 1 mm and used for chemical analysis. For each plot, a total of approximately 1000 cobs were collected and processed for chemical analysis, as described below. Triplicate 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