Introduction

Aflatoxins are a group of mycotoxins mainly produced by Aspergillus flavus and A. parasiticus (Keller et al., 2005; Yu et al., 2008). Although these two filamentous fungi have been known for a long time, their toxic secondary metabolites have been recognized only since the early 1960s (Hedayati et al., 2007). In 1976, the International Agency for Research on Cancer recognized aflatoxins as potential carcinogenic substances. Among the four major aflatoxins, aflatoxin B1, B2, G1, and G2, aflatoxin B1 is the most dangerous, being considered one of the most potent carcinogens ever discovered (Squire, 1981; Keller et al., 2005). More recently, other adverse effects of aflatoxins on the health of humans and farm animals have been described, including reduced weight gain, hemorrhaging, and suppression of the immune systems (Eaton, 1994). Aflatoxin contamination of agricultural commodities, including cereals, tree nuts, dried fruits, and other...
crops, is a worldwide problem, with higher incidence and severity in subtropical and warm temperate areas (Klich, 2007). Currently, approximately 100 nations in the world have established regulations for aflatoxins and other mycotoxins in food (CAST, 2003).

Corn (maize) is an important crop for food, animal feeding, biofuel, and other industrial applications. It is currently cultivated on a total area of 156 million hectares in approximately 100 countries within 40° S to 58° N latitude and all longitudes (Marshall, 2010; Nuss & Tanumihardjo, 2010). Aflatoxin contamination of corn is a concern in several agricultural areas, especially in developing countries where food safety measures are less stringent (Wu & Khlwangiset, 2010). A recent survey showed that approximately 4.5 billion people living in developing countries are chronically exposed to uncontrolled aflatoxin, which may exacerbate adverse health effects (Nuss & Tanumihardjo, 2010). Following the pioneering work of Anderson et al. (1975), it appears clear that aflatoxin contamination of corn mainly occurs during the growing season. A. flavus is a ubiquitous fungus, readily isolated from soil, crop residues, plant, air, and so on. Ecological studies have indicated that A. flavus is a common component of the soil microbial community (Abbas et al., 2009). Agricultural practices that promote the general health of corn, including planting time, irrigation frequency, crop rotations, and so on, have shown variable and unconvincing results in reducing aflatoxin contamination. Other than the level of initial inoculum, the intensity of aflatoxin contamination depends on a variety of other factors, including environmental conditions (i.e. daily temperatures, precipitations, etc.), insect damage, type of hybrid, and so on (Cleveland et al., 2003; Abbas et al., 2009). Not all A. flavus strains are capable of producing aflatoxins. This characteristic has been exploited by researchers to design biocontrol strategies. The basic concept of these biocontrol strategies consists of field application of nontoxicogenic strains to competitively exclude indigenous propagules from the soil (Abbas et al., 2009). This strategy was first applied to cotton and peanuts and more recently in corn (Dorner et al., 1992; Garber and Cotty, 1997; Dorner et al., 1999; Abbas et al., 2006). Propagules of A. flavus biocontrol strains are typically formulated as inoculated or spore-coated cereal grains (e.g. wheat, barley), thus facilitating field application and providing a food source for initial stages of fungal growth (Dorner et al., 2003; Abbas et al., 2006). In more recent years, a liquid formulation and a granular formulation based on bioplastic have been proposed as alternative approaches to deliver propagules of biocontrol strains of A. flavus (Accinelli et al., 2009; Lyn et al., 2009). This review article highlights progress in the use of bioplastic materials in the biocontrol of A. flavus.

Bioplastic and its applications

Since the 1940s, plastic has played a ubiquitous role in our civilization. Its versatility and ease of processing, has enabled plastic to penetrate every aspect of modern life. Approximately 140 million tons of petroleum-based polymers such as polyethylene, polypropylene, polystyrene, polyethylene terephthalate, polycarbonate, and polystyrene chloride are annually used in a variety of industrial and day-to-day plastic applications, including packaging, food industry, automotive industry, electronic devices, mulch films for agriculture, and so on (Keshavarz & Roy, 2010). Beside differences of physicochemical properties, a common aspect of these polymers is their recalcitrance to biodegradation. Although this characteristic is still important in many applications such as paints, other protective coatings, textiles, electrical insulation, and so on, it raises environmental concerns, which along with high crude oil prices have prompted much interest in biologically derived polymers, especially biodegradable polymers (Kumar et al., 1982; Philip et al., 2007; Kim & Dale, 2008; DiGregorio, 2009).

Biodegradable polymers with thermoplastic properties have been known for a long time. In the early 1920s, Maurice Lemoigne discovered that the soil bacterium Bacillus megaterium can produce quantities of an intracellular biopolyester, polyhydroxybutyrate (PHB). This discovery remained of academic interest until 1962, when Baptist and Laurel filed a patent claiming an industrial process for producing PHB using B. megaterium. Because of technical difficulties to obtain pure PHB biopolymer and poor mechanical properties of the derived plastic-like material, commercial interest of PHB lay dormant for approximately two more decades (Holmes, 1985). PHB belongs to the class of thermoplastic polymers known as poly(hydroxyalkanoates; PHAs), which has physical properties ranging from hard rigid solids to elastomers (Gunaratne & Shanks, 2008). Chemically, PHAs are polyesters of hydroxyalkanoic acids (Akaraonye et al., 2010). Polyhydroxybutyrate (PHB), the simplest natural form of PHA, is a polymer of (R)-3-hydroxybutyric acid produced by a wide variety of bacteria. Similarly to other members of the PHA class, PHB is accumulated as discrete granules within the bacterial cell. Depending on bacterial strains and fermentation conditions, a range of different PHAs can be produced. In the early 1980s, ICI Plastic Division started the industrial production of Biopol®, the copolymer of 3-hydroxybutyrate and 3-hydroxyvalerate (PHBV). PHBV has improved mechanical properties, including lower crystallinity and more elasticity than PHB (Holmes, 1985). Later, a variety of different PHAs-based products have entered the market, including everyday articles (i.e. beverage bottles, combs, etc.), medical applications (i.e. surgical sutures, slow release formulations for drugs, etc.), mulch films for agricultural crops, and so on. Although PHAs can also be chemically synthesized, microbial biosynthesis of this class of polymers remains the most convenient approach (Philip et al., 2007).

Another important biodegradable polyester is polylactide (PLA), which is currently used for manufacturing various products, such as biodegradable plastics and textile fibers. PLA is a polymer of lactide, a lactic acid diester.
Unlike the naturally occurring PHAs, PLA is a synthetic polyester, although the starting material, lactic acid, is found in nature (Stevens, 2003). The common industrial process for producing PLA starts with the fermentation of carbohydrates, mainly derived from hydrolyzed starch-rich resources (i.e. corn). The end product of bacterial fermentation, lactic acid is then separated from the cultivation media and chemically processed to produce PLA. Two major technical routes are used to produce PLA from the lactic acid: direct condensation polymerization of lactic acid and polymerization of a cyclic lactide dimer intermediate (Vink et al., 2003). The second route is the most widely adopted by industry (Vink et al., 2004). Nowadays, PLA is the most utilized biodegradable thermoplastic polymer (Rasal et al., 2010). Due to its biocompatibility in humans, PLA was initially used in biomedical applications such as sutures and prosthetic devices (Auras et al., 2008). In the last two decades, PLA-based products have replaced a variety of consumer applications (i.e. packaging materials, food and beverage containers, etc.).

Plants can synthesize numerous polymers, such as cellulose, starch, and many others. Starch is the second most abundant polysaccharide in nature after cellulose (Mooney, 2009). Over the last few years, there has been a renewed interest in biodegradable plastic and plastics made from annually renewable resources such as starch. Starch is composed of a mixture of linear and branched polysaccharides (Finkenstadt, 2005). As a natural and biodegradable polymer with potentially useful thermoplastic properties, starch has attracted attention for its use in the replacement of petroleum-derived synthetic materials particularly in the plastics industry (Otey et al., 1977; Koch & Ropper 1989; Fanta et al., 1992; Stenhousen et al., 1992; Evangelista et al., 1993; Swanson et al., 1993; Lawton and Fanta, 1994; Bastioli et al., 1994). A major limitation in the use of starch in the plastics industry is that the properties of native starches do not meet industrial needs. Thus, the functional properties of starches introduced to the commercial market are often modified by physical or chemical treatments to meet the needs of applications. Starch biopolymers are sensitive to water migration, and its polymeric glass transition temperature is relatively higher compared to petroleum-based polymers. Consequently, different plasticizers are added to reduce the moisture susceptibility and also to maintain polymeric flexibility in starch-based plastic applications. Different technologies for developing practical applications of starch-based plastics have been proposed over the years. However, blending starch with other compatible and biodegradable polymers is considered the most practicable approach (Stevens, 2003). A successful example of this approach is the starch-based bioplastic Mater-Bi® (MB) developed in the early 1980s by Novamont S.p.A. MB is a bioplastic product composed of starch, polycaprolactone, (ε-caprolactone) and a minor amount of a natural plasticizer (Bastioli, 2001). Different MB grades with a wide range of physical properties are currently available in the market. The basic industrial process for making MB is based on the destructuration of starch (getting rid of the granule structure) and combination with other polymers and plasticizers. MB is a readily adaptable product that is currently used for making shopping bags, biofillers, agricultural films, and a number of other commercial products (Bastioli, 2001). MB is completely biodegradable, having a rate of breakdown similar to that of cellulose (Bastioli, 1998).

Except for a small number of applications, including mulching films, horticultural containers, and plant clips, the use of bioplastic-based products in agriculture is still very limited. However, considering the increasing tendency to use green technologies in agriculture, the impact of bioplastic in this primary sector is expected to become much larger.

**Available technologies for the biocontrol of Aspergillus flavus**

Direct and indirect economic impact of aflatoxin contamination of corn and other commercially important crops (i.e. peanuts, cotton) has stimulated the research for strategies/technologies to control *A. flavus* in the field. It is widely recognized that aflatoxin contamination is frequently associated with plant stress (Payne et al., 1989; Tubajika & Damann, 2001). However, agronomical practices promoting plant health have a variable effect on aflatoxin contamination of corn (Klich, 2007; Abbas et al., 2009). Although major aspects of the ecology of *A. flavus* in the agroecosystem have been clarified, the level of aflatoxin contamination is not easily predictable and, even more importantly, not easily controlled. Many unpredictable factors are involved in the growth, soil colonization, and plant infection of the fungus (Payne et al., 2007; Zablotowicz et al., 2007; Abbas et al., 2008). In addition, changes in crop-management techniques (i.e. no-tillage systems, monoculture, adoption of Bt-protected corn) have further complicated the scenario (Wu et al., 2004; Accinelli et al., 2008a; Abbas et al., 2008, 2009). For instance, although some authors have demonstrated that Bt-transformed hybrids are effective in reducing aflatoxin contamination in corn (Dowd, 2003; Williams et al., 2003, 2005; Abbas et al., 2008), others have found no or inconsistent effects (Wiatrak et al., 2005; Bruns & Abbas, 2006; Pazzi et al., 2006; Abbas et al., 2007). A series of experiments conducted in the Mississippi Delta have shown that after corn harvest, aflatoxin levels remain lower in crop residues from a Bt corn hybrid during the whole intercropping period, compared to its non-Bt isolate. However, the population of *A. flavus* in soil/crop debris increases a similar amount during the intercropping season with both Bt and non-Bt corn (Accinelli et al., 2008a; Abbas et al., 2008).

In the last two decades, different approaches have been proposed to control aflatoxin contamination of corn. A promising approach is biocontrol or more
precisely the use of nontoxigenic strains of *A. flavus* to competitively displace indigenous aflatoxigenic isolates. This specific biocontrol technique is known as augmentative biological control, and in practice is based on the application of nontoxigenic strains of *A. flavus* to the soil of developing crops (Dorner, 2004; Abbas et al., 2008). An important prerequisite of any *A. flavus* biocontrol strain is that it should be both nontoxigenic and capable of competing with indigenous isolates (Dorner, 2004). *A. flavus* is capable of producing other toxins in addition to aflatoxins (i.e. cyclopiazonic acid) as well as producing toxic precursors of aflatoxins, including sterigmatocystin and related compounds, and the versicoloris (Cole & Cox, 1981). It is consequently highly recommended to use native and stable nontoxigenic biocontrol strains of *A. flavus*. Early studies conducted by Ehrlich (1987) and Brown et al. (1991) have demonstrated that inoculation of corn ears with nontoxigenic strains of *A. flavus* efficiently reduced aflatoxin levels of corn kernels. These positive results have stimulated more research on biocontrol of *A. flavus* in corn and other crops. Studies conducted in Southern United States have shown that direct application of propagules of nontoxigenic strains of *A. flavus* or *A. parasiticus* to emerged plants or to the soil results in significant reduction of aflatoxin contamination of peanuts (Dorner et al., 1992; Cotty & Bhatnagar, 1994). Beside the efficacy of this approach, high costs have limited its large-scale applications (Dorner, 1994). According to Daigle and Cotty (1995), a major difficulty in successful implementation of this biocontrol technique is the development of a reliable delivery system. As with other biocontrol microorganisms, in addition to having the intrinsic potential to serve as a biocontrol agent, an efficient formulation is essential for its functionality, practical use, and field performance (Butt & Copping, 2000). A simple approach for delivering propagules of biocontrol *A. flavus* strains consists of using inoculated cereal grains (Dorner, 2004; Abbas et al., 2008). After autoclaving, the grains (i.e. wheat, barley, rice) are inoculated with spore suspensions of nontoxigenic *A. flavus* isolates and incubated. After sufficient fungal growth has occurred (5–10 days), grains are dried and directly used or stored at 4–5 °C to preserve fungal viability. Once applied to the field soil, the fungus rapidly resumes growth and sporulation. Other approaches have been explored for delivering biocontrol strains of *A. flavus*. A technique widely adopted for formulating biocontrol fungal agents and consisting of entrapment of fungal propagules in alginate pellets was evaluated by Daigle and Cotty (1997) for delivering conidia of a nontoxigenic strain of *A. flavus*. The authors demonstrated the practicability and efficiency of this formulation in laboratory studies. However, for a number of reasons, this technique has not been further implemented for field applications. A variation of alginate pellets was later proposed by Daigle et al. (1997) and now known as Pesta. In Pesta pellets, conidia are entrapped in a wheat gluten matrix containing kaolin. A major advantage of this formulation is based on the high content of starch which provides a suitable food source to the biocontrol fungus. Similar to alginate pellets, the use of Pesta granules has remained limited primarily to laboratory and greenhouse studies. High production costs for making alginate and Pesta pellets are major constraints to their field-scale use.

Nowadays, the use of cereal grains is the preferable delivery approach for field applications of nontoxigenic strains of *A. flavus*. This approach has been further simplified with respect to that described above. In 2001, Cole and Dorner (2001) developed a technique in which conidia of a biocontrol *A. flavus* strain are coated on to the surface of hulled barley grains. More specifically, conidia are first suspended in vegetable oil and then sprayed on the surface of grains. Addition of diatomaceous earth absorbs remaining oil. The final product is applied to the field using on-farm granular application equipment. As opposed to the initial proposed technique (inoculated grains), this process does not require sterilization and incubation of the grains, which thus further reduces production costs. This coated hulled-barley formulation was approved by the EPA for registration for the biocontrol of *A. flavus* in peanut in 2004. Originally developed by USDA-ARS, this formulation was initially produced and commercialized by the Georgia-based company Circle One Global under the trade name Afla-Guard®. In 2009, Afla-Guard® received the EPA approval for the use in corn and its license has been acquired by Syngenta Crop Protection. In the meantime, research continues in the effort to find other solutions to the aflatoxin problem. Recently, Lyn et al. (2009) have proposed a liquid formulation for direct spray application of a nontoxigenic *A. flavus* strain. Field studies conducted in Southern United States using this clay-based water dispersible granular formulation have demonstrated its efficiency in controlling aflatoxin contamination of corn.

**Applications of bioplastic in the biocontrol of *Aspergillus flavus***

During the last decade, an increasing number of bioplastic-based products have been launched in the market. Most of these applications have simply replaced already existing petroleum-derived plastic products (i.e. single-use disposable plates, shopping bags, accessories for household appliances, etc.); Song et al., 2009; Akaraoeye et al., 2010). However, as stated before, only a limited number of bioplastic products have been specifically developed for agricultural applications. Considering that most of the polymers currently used in the bioplastic industry are derived from annually renewable agricultural resources (i.e. simple sugars, starch, etc.), the use of bioplastic in the production and management of crops would be consistent with the concept of sustainable agriculture. Although the term *bioplastic* does not necessarily implicate biodegradability of the material itself, most of the bioplastic-based products that have entered in the market are specifically designed to satisfy this important
requirement (Stevens, 2003). Obviously, a major advantage of these products is their biodegradability which implies that a wide range of environmental microorganisms have the capability to use bioplastic as a source of nutrients, mainly as carbon sources, for sustaining their growth. The application described below simply inverts this basic elementary concept. Since several microorganisms, especially filamentous fungi, are capable of growth on bioplastic matrices, why not use a bioplastic-based formulation for field application of biocontrol fungi? The concept implies that the bioplastic matrix would not only serve as a carrier for beneficial microorganisms (i.e. biocontrol fungal agents), but also may provide a food source, thus facilitating the initial stages of their growth (Accinelli et al., 2008b). This latter aspect is particularly important in determining the success of augmentative biocontrol strategies based on the use of biocontrol fungi.

As with other filamentous fungi, A. flavus is commonly cultured onto a carbohydrate-rich medium such as synthetic potato dextrose. For mass production of A. flavus biomass (mycelium or conidia), less expensive media can be used (e.g. homemade potato dextrose, starchy agricultural raw materials). As a colonizer of starch-rich seeds (corn, rice, etc.), A. flavus is able to hydrolyze starch (Mellon et al., 2005). It is not surprising, then, that cereal grains have been widely used for delivering biocontrol A. flavus strains to field soil and that Afla-Guard® (Dorner, 2004; Cotty et al., 2007). As described above, starch is the major component of the bioplastic MB. Consequently, we explored the feasibility of this bioplastic-based formulation to replace grain seeds for delivering and facilitating soil colonization of a biocontrol A. flavus strain.

Early laboratory studies demonstrated that spores of the nontoxigenic strain NRRL 30797 are easily entrapped on MB bioplastic granules, giving a final potency (density of microbial propagules) of approximately 7.0 cfu g⁻¹. When stored at a cool temperature, potency of the bioplastic-based formulation remains approximately constant for 3 months. After hydrating, spores rapidly germinate and an intense growth of the fungus is observed (Figure 1). Using different approaches, including conventional (plate count) and molecular methods (end-point PCR, quantitative PCR, and DNA fingerprinting), our laboratory studies clearly showed that the bioplastic-based formulation is effective in delivering the biocontrol fungus and supporting initial stages of its growth (Figure 2; Accinelli et al., 2009). These laboratory-scale findings were then confirmed by a 2-year field study conducted in Northern Italy. Bioplastic granules entrapping spores of the nontoxigenic strain A. flavus NRRL 30797 were applied in May, approximately 1 month after corn planting, at the rate of 15 and 30 kg ha⁻¹. The latter dosage gave better results in controlling aflatoxin contamination, with a reduction of more than 80% with respect to the untreated control (Accinelli et al., 2011). The study also showed that the replacement of indigenous aflatoxin isolate was rapid and intense.

MB granules are industrially produced from renewable sources (corn starch). Beside the favorable environmental profile, bioplastic granules offer a series of practical advantages, including easy handling and field application (Accinelli et al., 2009). It is well known that a major criticism of agricultural biocontrol strategies is their complexity and low technology input. The
bioplastic-based formulation described here would open the exploration of bioplastic materials for biocontrol of agricultural pests.

As indicated above, the MB bioplastic matrix promotes vigorous growth and sporulation of the fungus. This property can also have other practical implications. For instance, we speculated that the starch-based bioplastic would serve as a baiting material for isolation of Aspergilli. In a study conducted using soil and corn kernel samples, we evaluated the feasibility of this approach to recover Aspergilli. After impregnation of bioplastic granules (for corn kernels) or rods (for soil) with the A. flavus selective medium, modified rose bengal (Abbas et al., 2004), the resulting formulations were incubated with samples of soil or corn kernels at room temperature for 10–14 days. Visual observation and incubating of baiting granules or rods on PD agar clearly showed their selectivity for isolating Aspergilli (Figure 1). These findings were also confirmed by sequencing DNA fragments of recovered fungal isolates. When granules used for baiting Aspergilli from kernel samples were incubated in test tubes containing yeast sucrose medium and then analyzed for aflatoxin concentrations, we found a significant correlation between the amount of aflatoxin produced by baited fungi and aflatoxin contamination of corn kernels. This simple and cost-effective bait system could help with monitoring Aspergilli from soil and corn kernels. It is widely recognized that in developed countries, because of well-controlled storage conditions, aflatoxin level of corn remains approximately unchanged during storage. Conversely, corn storage under poorly controlled and far from ideal conditions, as often seen in developing countries, can result in additional fungal proliferation and aflatoxin production (Shier et al., 2006). In these cases, the availability of a rapid tool for monitoring this risk would be helpful.

Despite the proposition of various baiting approaches for detecting plant fungal pathogens in soil or other plant-growth media, those most widely used are based on conventional plate counting techniques (Paulitz & Schroeder, 2005; Eguchi et al., 2009). In recent years, the use of DNA-based methodologies has been proposed (Nechwatal et al., 2001; Matsumoto, 2003). However, these methods are based on endpoint PCR approaches and do not permit the specific quantification of the target fungus. Considering the importance of monitoring a specific microorganism, and the wide acceptance of quantitative PCR protocols, the possibility of coupling a simple baiting system to a DNA-based quantification protocol would have practical implications in fungal pathogens tracking and quantification. Unlike baiting materials previously proposed by other scientists, such as autoclaved cereal seeds, which are naturally contaminated by microorganisms and can produce false-positive samples, bioplastic rods or granules are made of DNA-free matrices and, after extrusion at high temperatures (>160 °C), they are autoclaved for a long time thus further reducing the occurrence of contamination.

Conclusions

Direct and indirect costs associated with aflatoxin contamination of agricultural crops have stimulated the development of strategies and techniques for managing the problem. Because the importance of avoiding proliferation of aflatoxin-producing fungi, mainly A. flavus and A. parasiticus, during storage of agricultural commodities, strategies relying on preharvest management of these fungi have prompted increasing interest in the last decades. Available published data indicate that among the different strategies, biocontrol is the most effective approach for controlling aflatoxin contamination in crops such as peanuts, cotton, and corn. Biocontrol of aflatoxins is based on field application of propagules of competitive nontoxicogenic strains of A. flavus to competitively displace indigenous toxigenic soil isolates. These biocontrol strains are typically applied to agricultural fields as inoculated or spore-coated grain seeds. Increasing knowledge in formulation of delivery vehicle for fungal biocontrol agents and the availability of new materials and technologies, has resulted in the development of other practical solutions, including a granular bioplastic-based formulation. This latter formulation was first proposed in 2008 and is still under evaluation and improvement. However, bioplastic granules entrapping the biocontrol strain A. flavus NRRL 30797 has been shown to serve as an efficient tool for delivering the fungus and to facilitate its soil colonization. Initial field experiments conducted in Northern Italy using this technology produced encouraging results. This starch-based bioplastic can also be used as a tool for selective isolation of A. flavus from soil and corn kernel samples.

Acknowledgements

The authors want to express their deep gratitude to Dr. Robert M. Zablottlewich for his valuable contribution in the development of the bioplastic formulation described here in this manuscript. The authors greatly appreciate his enthusiasm and support with this project.

Declaration of interest

The authors declared no conflict of interest.

References


