



## Use of a granular bioplastic formulation for carrying conidia of a non-aflatoxigenic strain of *Aspergillus flavus*

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### ARTICLE INFO

#### Article history:

Received 3 November 2008

Received in revised form 3 March 2009

Accepted 4 March 2009

Available online 5 April 2009

#### Keywords:

Biocontrol formulations

Bioplastic

Mycotoxins

Biopesticides

### ABSTRACT

Previous research demonstrated that aflatoxin contamination in corn is reduced by field application of wheat grains pre-inoculated with the non-aflatoxigenic *Aspergillus flavus* strain NRRL 30797. To facilitate field applications of this biocontrol isolate, a series of laboratory studies were conducted on the reliability and efficiency of replacing wheat grains with the novel bioplastic formulation Mater-Bi<sup>®</sup> to serve as a carrier matrix to formulate this fungus. Mater-Bi<sup>®</sup> granules were inoculated with a conidial suspension of NRRL 30797 to achieve a final cell density of approximately log 7 conidia/granule. Incubation of 20-g soil samples receiving a single Mater-Bi<sup>®</sup> granule for 60-days resulted in log 4.2–5.3 propagules of *A. flavus*/g soil in microbiologically active and sterilized soil, respectively. Increasing the number of granules had no effect on the degree of soil colonization by the biocontrol fungus. In addition to the maintenance of rapid vegetative growth and colonization of soil samples, the bioplastic formulation was highly stable, indicating that Mater-Bi<sup>®</sup> is a suitable substitute for biocontrol applications of *A. flavus* NRRL 30797.

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### 1. Introduction

Augmentative biological control is as a pest management tactic that utilizes the deliberate introduction of living natural enemies to lower the population level of invasive pests (DeBach and Rosen, 1991). Biological control has been utilized for more than 100 years in efforts to control a wide number of agricultural pests including fungi, insects and weeds (Siddiqui and Mahmood, 1996; Stiling and Cornelissen, 2005). Biocontrol strategies have been implemented to control aflatoxin contamination in several important agricultural crops, such as peanut, cotton and corn (Abbas et al., 2006; Cotty, 1994; Dorner et al., 1992). Aflatoxins are highly carcinogenic secondary metabolites produced by several species of *Aspergillus* section *flavi*, including *A. flavus* link, *A. parasiticus* Speare, and *A. nomius* Kutzman, Horn and Hesseltime. Of these, *A. flavus* is the most abundant aflatoxin-producing species associated with corn (Abbas et al., 2004a,b; Abbas et al., 2008; Wicklow et al., 1998). *Aspergillus flavus* is readily isolated from diverse environmental samples; however, soil and crop residues are considered the natural habitat of this fungus (Abbas et al., 2008). In addition, soil serves as a reservoir of conidia or sclerotia (spores) which can infect susceptible crops (Scheidtger and Payne, 2003).

Management of aflatoxin-producing fungi in corn is a difficult task, requiring an integrated approach including optimization of agronomical practices (i.e. irrigation, fertilization, optimized planting date, etc.), and appropriate hybrids. These practices promote the general health of corn and can reduce, but not eliminate, aflatoxin contamination (Cleveland et al., 2003). Consequently, there is a need for additional, practical and cost-effective strategies to limit aflatoxin contamination of corn (Cleveland et al., 2003). One attractive option to supplement, but not supplant, these agronomical practices is biological control. Most aflatoxin biological control programs can truly be defined as bio-competition since they do not utilize parasites or diseases of the pest, but instead use atoxigenic *Aspergillus* spp. to competitively exclude toxigenic fungi.

Surveys conducted in multiple geographical regions have found that not all strains of *A. flavus* produce aflatoxins (Abbas et al., 2004a; Cotty and Bhatnagar, 1994). It is therefore not surprising then, that current biocontrol strategies rely upon the ability of non-aflatoxigenic strains to competitively exclude indigenous aflatoxin-producing *Aspergilli* (Cleveland et al., 2003). Successful reduction of aflatoxin contamination through the introduction of competitive non-aflatoxin producing strains of *A. flavus* has been demonstrated in a number of crops, including corn (Dorner et al., 1992; Dorner, 2005). Biocontrol fungi can be applied to soil as alginate pellets, pre-gelatinized starch-flour granules, or colonized grains (Dorner, 2008; Honeycutt and Benson, 2001; Lewis et al., 1998). Among some of the successful formulations, a pasta-like

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product (Pesta) and a coated hulled-barley formulation (Afla-Guard®) have proven to be effective for delivering non-aflatoxigenic strains of *A. flavus*, as well as, other biocontrol fungi (Connick et al., 1998; Dorner, 2008; Singh et al., 2007). Application of these protocols has also been shown to be highly effective in recent studies conducted by the USDA-ARS at the Stoneville (Mississippi) research station. In these efforts, Abbas et al. (2006) demonstrated that aflatoxin contamination in corn is dramatically reduced by field application of non-aflatoxin producing *A. flavus* strain NRRL 30797. In Abbas et al. (2006) conidia of the competitive non-aflatoxigenic strain were applied to soil as inoculated wheat grains. To facilitate handling, long term storage and field application of the *A. flavus* NRRL 30797 biocontrol isolate, we have evaluated a novel granular formulation consisting of the commercial bioplastic Mater-Bi® (Novamont S.p.A., Novara, Italy).

Mater-Bi® (MB) is a bioplastic product composed of starch, polycaprolactone ( $\epsilon$ -caprolactone) and a minor amount of a natural plasticizer (Bastioli, 2001). MB is a reliable and 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). In addition to the highly favorable low environmental impact profile, its physical properties facilitate product handling and field application. These properties make granular MB an excellent candidate for biocontrol applications of *Aspergillus* propagules. Therefore, we report a series of studies evaluating the reliability of MB granules as substrate for delivering conidia of the non-aflatoxigenic strain of *A. flavus* NRRL 30797 in soil.

## 2. Methods

### 2.1. Soil and fungal strains

The soil used in this study was collected on December 2007 from a 1-ha uncropped area located at the experimental farm of the University of Bologna, Italy. This soil is classified as a Cataldi silty loam (Udic Ustochrepts, fine silty, mixed, mesic) with 380 g kg<sup>-1</sup> sand, 245 g kg<sup>-1</sup> clay, 375 g kg<sup>-1</sup> silt, 8.5 g kg<sup>-1</sup> organic C, and pH (1:2.5 soil/water mixture) of 8.0. A total of ten soil subsamples (5–20 cm depth) were arbitrarily collected using a sterilized spatula. These subsamples were bulked together, homogenized by passing through a 4-mm sieve, and stored at 4 °C until processed. A sufficient mass of soil was also autoclaved at 120 °C for 60 min on three successive days.

Two strains of *A. flavus* were selected for this study; the non-aflatoxigenic strain *A. flavus* NRRL 30797 (K49) and the aflatoxin-producing *A. flavus* NRRL 30796 (F3W4). Properties of the two strains have been previously described (Abbas et al., 2006; Accinelli et al., 2008). Fresh conidia were generated by plating 10<sup>8</sup> spores ml<sup>-1</sup> on acidified potato dextrose agar (PDA) and incubating at 37 °C for 5–7 days. Conidia were recovered from plates in 5 ml of sterile 0.2% Tween 20 collected by gentle scrapping. Conidia concentration was determined by microscopic counts using a hemocytometer and conidial densities adjusted as necessary.

### 2.2. Preparation of MB granules and soil samples incubation

Granules of Mater-Bi® ZF03U/A (MB) with average size of 5 mm long and 3 mm diameter were supplied by Novamont S.p.A. (Novara, Italy). Conidia from the *A. flavus* NRRL 30797 were entrapped within the MB granules by equilibrating the granules in conidial suspensions ranging from log 5.7 to log 8.7 conidia ml<sup>-1</sup> with shaking (300 rpm) for 4-h. After this incubation, the conidial suspensions were then forced through these impregnated granules

using a piston-like device at a pressure of 62 kPa (Riff98, Italy). Finally, granules were dried at 40 °C for 2 h and surface cleaned by flushing compressed air generated from a commercial air-compressor. The total number of entrapped *A. flavus* conidia in the granules was evaluated by plate count. Evaluations of colony forming units on granular surface, shelf life of granules, and MB ability to support growth and sporulation were determined as described below.

Estimates of the percentage of *A. flavus* conidia located on the granule surface were determined by exposing the granules to UV rays for 60 min using a model G20T10 UV germicidal lamp (Sankyo Denki, Japan). Difference in the total colony forming units (cfu) of unexposed and UV exposed granules allowed an estimation of the propagules present on the granule surface after air-pressure cleaning. For shelf life evaluation, granules were transferred into zip-lock plastic bags (1-l volume) and stored in the dark at 5 and 25 °C. After 3 and 6 month-storage, colony viability was evaluated by plate count. The potential of MB matrix to support *A. flavus* growth and sporulation was evaluated by inoculating 200 untreated pre-wetted MB spherical granules (2 mm diameter) with 1.5 ml of a *A. flavus* NRRL 30797 conidial suspension (log 2.7 conidia ml<sup>-1</sup>) and incubating on Petri plates (45 mm) at room temperature. At selected intervals, fungal colonization of MB granules was estimated by visual observation and quantitative PCR (qPCR).

To estimate soil colonization rate, soil samples were prepared by weighing 20 g of soil in 50-ml sterilized screw-top tubes and moisture adjusted (sterilized distilled water) to the gravimetric content at -33 kPa. Freshly prepared MB granules (1, 2, or 3) were transferred to tubes and samples were homogenized by gently mixing. The samples were incubated in the dark at 25 °C for up to 60 days with soil moisture maintained by weighing samples every 7 days and adding water as needed. At selected intervals, triplicate samples were removed and *A. flavus* colonization estimated by plate count and qPCR. Samples containing three MB granules were also used for estimating biodegradation rate of granules in soil by measuring their weight loss during the 60-day incubation period. To remove soil attached to the granule surface, granules from each sample were transferred to microcentrifuge tubes containing distilled water and glass beads (425–600  $\mu$ m; Sigma-Aldrich, Germany). Samples were vortexed for 5 min and the slurry removed from tubes to recover the MB granule. The washing process was repeated if required. The washed granules were dried at 40 °C for 2 h and weighed.

To assess the ability of *A. flavus* strain NRRL 30797 introduced as MB granules to compete with soil populations of *A. flavus*, experiments were conducted with native and sterile soil with an artificially established population of the aflatoxigenic *A. flavus* strain NRRL 30796. To establish the *A. flavus* populations, the soil samples were inoculated with NRRL 30796 conidia and incubated for 2 months to establish a population of  $\sim$ log 2.9 cfu g<sup>-1</sup> soil. As in the soil colonization study, one to three MB granules inoculated with the strain NRRL 30797 were introduced in each 20-g soil sample. Samples were incubated as previously described and relative abundance of toxigenic isolated was estimated as described below.

The carrying capacity of MB granules to maintain viable *Aspergillus* conidia was evaluated relative to another starch-based formulation, Pesta. Pesta is a wheat gluten-kaolin matrix encapsulating fungal propagules. Pesta granules were prepared as described in Connick et al. (1998) with minor modifications. Briefly, 32 g of semolina (Barilla, Italy), 8 g of kaolin (Merck, Germany) and 21 ml of a conidia suspension (strain NRRL 30797) were manually kneaded to make dough. The dough was extruded through a Rosle potato masher and cut to obtain cylindrical granules of the same size of MB granules. After drying at 40 °C for 2 h, Pesta granules were transferred into screw-top tubes and stored

in the dark at 5 and 20 °C. Soil samples containing a single Pesta granule were prepared and incubated as described for MB granules.

### 2.3. Enumeration of *Aspergillus flavus* propagules in granules and soil samples

Enumeration of *A. flavus* conidia present in MB and Pesta granules was conducted by placing single granules in centrifuge tubes containing 10 ml of phosphate buffer saline (PBS) and 5 g of glass beads. The *A. flavus* were recovered following vortexing (5 min.) followed by agitation (2 h at 200 rpm). This suspension was serially diluted in PBS prior to plating. Suspensions were diluted in triplicate and 100- $\mu$ l aliquots were spread onto modified dichloronitro-aniline rose bengal agar (MDRBA) as per Abbas et al. (2004b, 2006). Plates were incubated at 37 °C for 4–5 days and the resulting *A. flavus* colonies were recorded. For enumeration of total culturable fungi in MB and Pesta granules, MDRBA was replaced with acidified potato dextrose agar and incubated at 25 °C.

To determine the number of *A. flavus* propagules in soil and the relative abundance of atoxigenic isolates, the method described in Abbas et al. (2004b) was used with minor modifications. Briefly, soil samples (10 g) were suspended in a 90-ml water agar solution (0.2%), vortexed for 3 min, and shaken for 1 h at 250 rpm. This suspension was used for ten-fold serial dilutions prepared in PBS, and 100- $\mu$ l aliquots were plated onto MDRBA and incubated at 37 °C for 4–5 days. A sufficient number of colonies (10–30) of *A. flavus* were randomly selected and transferred to  $\beta$ -cyclodextrin (0.3%) potato dextrose agar; the new plates were incubated at 28 °C for 5 days in the dark. Aflatoxin-producing isolates were identified as colonies that displayed blue fluorescence during exposure to UV light (365 nm). Aflatoxin production was also confirmed by observing color change of isolates after exposure to aqueous ammonium hydroxide (27% v/v) for 30 min.

### 2.4. DNA analysis

A series of DNA-based methods were used for tracking the introduced *A. flavus* strain NRRL 30797 in incubated soil samples. These include PCR with known aflatoxin biosynthetic gene primers, qPCR of the biosynthetic gene *omtB* (*aflO*), and PCR of internal transcribed spacers.

PCR-based detection of *A. flavus* DNA in soil was conducted using the procedure described in Accinelli et al. (2008). Briefly, total soil DNA was isolated using the commercial kit PowerSoil (Molecular Bio Laboratories Inc., Solana Beach, CA) and purified with the

Wizard DNA Clean-Up System (Promega, WI), following the instructions of the manufacturers. Eluted DNA was used for PCR analysis for detecting five genes of the aflatoxin biosynthesis pathway (Table 1). The PCR reaction mixture contained 25  $\mu$ l of RedTaq ReadyMix (Sigma–Aldrich), 0.5  $\mu$ M of each primer (Operon Biotechnologies, Germany), 5–10 ng template DNA and water to a final volume of 50  $\mu$ l. The cycling was performed with the T3 DNA thermalcycler (Biometra, Germany) as follows: 94 °C (4 min) followed by 30 thermal cycles of 94 °C (30 s), 56 °C (30 s), 68 °C (60 s), and a final elongation step at 72 °C for 15 min. The amplified products were separated on a 1% agarose gel and visualized by staining with SYBR Green I (Sigma–Aldrich).

Total *A. flavus* DNA in incubated soil samples was estimated by qPCR. In addition, a selected number of samples were also analyzed to quantify total *A. flavus* DNA remaining in MB granules during the 60-day incubation period. Soil DNA was isolated using the same procedure adopted for PCR analysis. DNA isolation from MB granules was performed following the CTAB procedure, with minor modifications (Doyle and Doyle, 1990). Briefly, two MB granules were removed from soil samples, dried at 40 °C for 2 h, vortexed for 5 min to remove adhering soil and air-flushed by high-pressure air. Surface-cleaned granules were transferred to a 2-ml microcentrifuge tube containing 500  $\mu$ l of CTAB buffer and glass beads (425–600  $\mu$ m; Sigma–Aldrich). After vortexing for 2 min, tubes were incubated at 65 °C for 15 min, and an equivalent volume of chloroform:isoamyl alcohol (24:1) was added to tubes. Tubes were gently shaken and centrifuged at 10,000g for 5 min before the addition of 2/3 volume of isopropanol/ammonium acetate to precipitate the DNA. The pellet was rinsed with 70% ethanol, air dried and resuspended in 100  $\mu$ l of TE buffer.

DNA amplification was performed using a primer set targeting the aflatoxin cluster gene *omtB* (*aflO*) (Table 1). Efficiency of qPCR was tested by including amplification of the *laeA* gene, a global regulator gene of the secondary metabolism in Aspergilli, as indicated in Kim et al. (2008). DNA isolated from soil samples treated with 10-fold dilutions of *A. flavus* NRRL 30797 conidial suspension, as described above, was utilized as template for 25- $\mu$ l qPCR reactions. Each 25- $\mu$ l qPCR reaction contained 2  $\mu$ l of DNA, 12.5  $\mu$ l of 2x TaqMan Universal PCR Master Mix (Applied Biosystems, CA), and 0.2  $\mu$ M of each primer. Thermocycling conditions were as follows: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The resulting samples were analyzed using an ABI Prism 7700 Sequence Detection System (Applied Biosystem). After quantification, amplified fragments samples were subjected to melting-curve analysis. A standard curve was generated by plotting cycle threshold values (Ct) against logarithmic-transformed

**Table 1**

Primer sequences of genes tested by PCR and qPCR. Genes are abbreviated using nomenclature found in the corresponding citation that primers were selected from. Expected product size are expressed in base pairs (bp).

Gene	Primer sequence	PCR product size (bp)	References
<i>aflD</i> - F	5'-ACGGATCACTTAGCCAGCAC-3'	990	Scherm et al. (2005)
<i>aflD</i> - R	5'-CTACCAGGGGAGTTGAGATCC-3'		Scherm et al. (2005)
<i>aflO</i> - F	5'-GCCTTGACATGGAACCATC-3'	1330	Scherm et al. (2005)
<i>aflO</i> - R	5'-CAAAGATGGCCTGCTCTTTA-3'		Scherm et al. (2005)
<i>aflP</i> - F	5'-GCCTTGCAAACACACTTTCA-3'	1490	Scherm et al. (2005)
<i>aflP</i> - R	5'-AGTTGTTGAACGCCCACT-3'		Scherm et al. (2005)
<i>aflQ</i> - F	5'-CGACTGTTGGCCTTTTCATT-3'	1088	Scherm et al. (2005)
<i>aflQ</i> - R	5'-ATAGCGAGGTTCCAGCGTAA-3'		Scherm et al. (2005)
<i>aflR</i> - F	5'-CGAGTTGTGCCAGTTCAAAA-3'	999	Scherm et al. (2005)
<i>aflR</i> - R	5'-AATCTCGCCCACTACTA-3'		Scherm et al. (2005)
ITS1 - F	5'-TCCGTAGGTGAACCTGCGG-3'	~1100	O'Donnell (1993)
ITS4 - R	5'-GGTCCGTGTTCAAGACGG-3'		O'Donnell (1993)
<i>omtB</i> - F	5'-AAGCAGATCATCCAGTGAT-3'	~130	Kim et al. (2008)
<i>omtB</i> - R	5'-CGAGTTGTGCCAGTTCAAAA-3'		Kim et al. (2008)
<i>laeA</i> - F	5'-GCTGGTACAATTGGCTGTC-3'	~130	Kim et al. (2008)
<i>laeA</i> - R	5'-CCCTCCGACTTGACTTCTG-3'		Kim et al. (2008)

amounts of target DNA obtained from 10-fold dilutions of DNA isolated from *A. flavus* NRRL 30797. Correlation between amount of target DNA recovered from soil and the size of *A. flavus* propagules (estimated by plate count) was calculated.

At the end of the 60-day incubation period, the diversity of the soil fungal community was estimated by rDNA internal spacer analysis (RISA). Total soil DNA was isolated as described previously and amplified using the universal fungal primers ITS1/ITS4 targeting the internal transcribed (ITS) region and the PCR protocol previously described (Table 1). Ten microliters of PCR products were digested with 10 units of *Hae* III in a total volume of 25  $\mu$ l at 37 °C for 2 h, and the digested products were separated by vertical nondenaturing 8% polyacrylamide gel electrophoresis and visualized by SYBR Green I staining.

### 2.5. Statistical analysis

All experiments were conducted in triplicate and data were subjected to analysis of variance. Mean values were compared using Tukey's HSD test and significant differences were detected at the  $P = 0.05$  level. The number of *A. flavus* propagules derived from soil or MB granules were expressed as the log (10) transformed colony forming units (cfu)  $g^{-1}$  dry weight.

## 3. Results and discussion

### 3.1. Practical aspects and shelf life of MB granules

Equilibrating MB granules with conidia suspensions ranging from log 5.7 to 8.7 conidia  $ml^{-1}$  resulted in a range of propagules of log 4.3 to 7.0 cfu  $g^{-1}$ , respectively (Fig. 1). Considering that effective biocontrol formulation depends on the potency (number and ability to produce propagules) we focused on the MB granules generating the highest propagule rate (i.e. highest potency). Another essential prerequisite for any biopesticide is that it should be safe for operators. Even though *A. flavus* NRRL 30797 has been shown to produce no toxins (aflatoxins or cyclopionic acid, Abbas et al., 2006), our efforts were focused on producing a solid formulation with fungal propagules mostly entrapped within the granules. This will reduce risks of mold inhalation during handling and field application. Exposing granules to germicidal UV light led to only a 25% decrease of recoverable propagules, thus confirming that most of the conidia are located within the granules (data not shown).

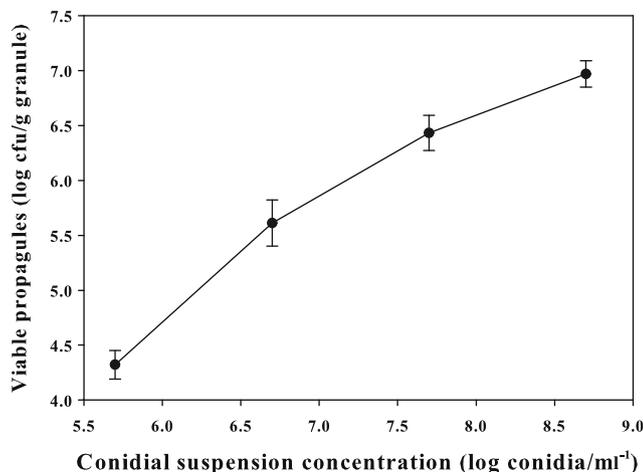


Fig. 1. Effect of conidial suspension concentration on final potency of Mater-Bi<sup>®</sup> granules. Each point represents mean  $\pm$  STD ( $n = 3$ ).

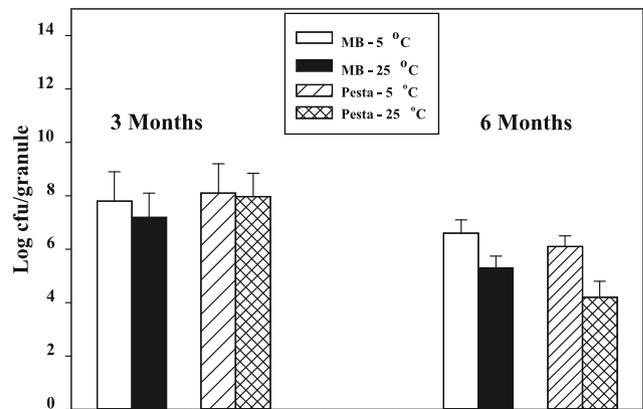


Fig. 2. Effect of temperature and storing time on shelf life of Mater-Bi<sup>®</sup> and Pesta granules. Initial potency of MB and Pesta granules was of log 8.7 cfu per granule. Each bar represents mean  $\pm$  STD ( $n = 3$ ).

As shown in Fig. 2, following storage of inoculated MB granules for six months at 5 and 25 °C the number of viable *A. flavus* NRRL 30797 propagules declined approximately 75% and 95%, respectively. No significant decline was observed in MB granule stored for a shorter period (three months) at either temperature. As expected, MB granules maintained their physical integrity over the whole six month storing period (Fig. 3). This is consistent with the physical properties of MB, which are similar to those of traditional petrol-based plastic matrices (Bastioli, 1998). Pesta granules showed a comparable shelf life to MB granules (Fig. 2), showing declines of approximately 90% and 60% for 5 and 25 °C at 6 months, respectively. Beside intrinsic factors influencing conidia viability, Pesta granules are made of friable semolina-based materials which results in readily breakable product (Fig. 3). This implicates that a decrease of the initial product potency caused by handling, storing and field application could not be excluded. In contrast, MB granules are highly resilient to these same types of damage, and thus need no particular precautions to prevent loss of potency.

The biodegradation dynamics of MB granules in soil is depicted in Fig. 4. Approximately 40% of the granule weight was lost during the 60-day incubation period. As expected, Pesta granules exhibited a more rapid degradation. After a 10-day incubation, Pesta granules were almost completely disintegrated; thus it was not possible to accurately measure their weight. The biodegradation rate of MB granules reported here is comparable with those reported by other authors (Kim et al., 2000; Mezzanotte et al., 2005; Rutkowska et al., 2004).

Research has shown that *Aspergilli* can grow on a wide range of carbon-rich substrates, including bioplastic materials (Bergenholtz



Fig. 3. Pesta (left) and Mater-Bi<sup>®</sup> (right) granules after six month of storing in plastic bags.

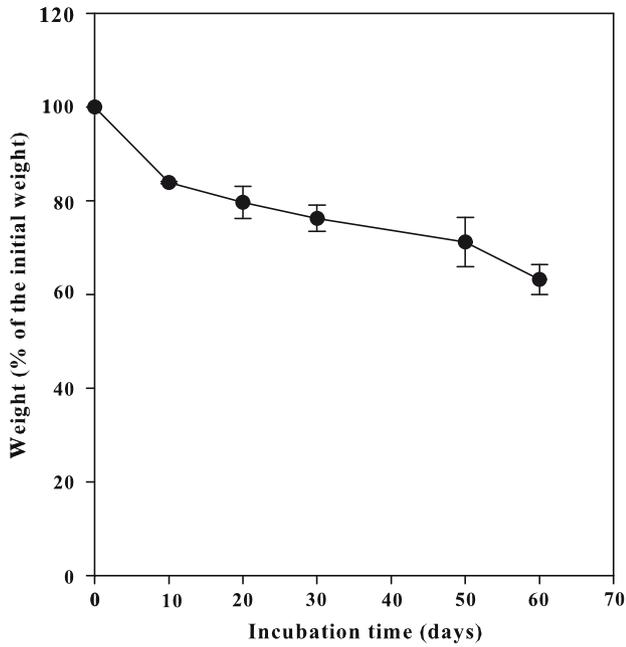


Fig. 4. Biodegradation of Mater-Bi® granules in soil expressed as percentage of the initial weight. Each point represents mean ± STD (n = 3).

and Nielsen, 2002; Kim et al., 2000). Quantitative PCR analysis and visual observation demonstrated that MB granules adequately supported fungal growth (Table 2; Fig. 5). In contrast to Pesta, no detectable levels of unwanted fungi were found in NRRL 30797-inoculated MB granules (data not shown). Pesta is a wheat flour-based product that may contain abundant fungal contaminants. This is an aspect of great importance due to the fact that these contaminating fungi have the potential to be mycotoxin producing fungi (*Aspergillus* spp., *Fusarium* spp. etc.).

### 3.2. Dynamics of *Aspergilli* colonization of soil

The dynamics of soil colonization by the introduced *A. flavus* in MB granules is shown in Fig. 6. Plate counts and PCR analysis showed detectable levels of *A. flavus* by the first sampling time (10 days of samples incubation) (Figs. 6 and 7). The total size of *A. flavus* population increased during the first month of sample

Table 2

Total amount of *A. flavus* NRRL 30797 DNA isolated from incubated Mater-Bi® granules. Number is means of three replicates ± STD.

0 days	5 days	15 days
Amount of target DNA ( $\times 10^3$ fg g <sup>-1</sup> )		
12.43 ± 3.95	62.11 ± 6.23	127.99 ± 8.97

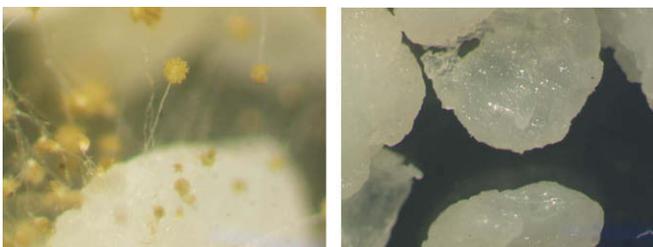


Fig. 5. Growth of *A. flavus* NRRL 30797 on inoculated Mater-Bi® granules (1–2 mm diameter) (left) and in untreated (control) granules (right).

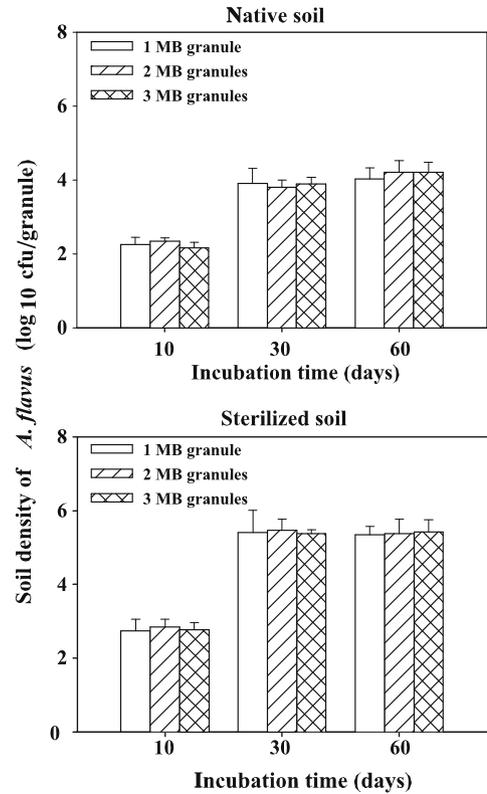


Fig. 6. Colonization of native and sterilized soil by *A. flavus* NRRL 30797 introduced as Mater-Bi® (MB) granules. Each point represents mean ± STD (n = 3), background propagule density of *A. flavus* in non-inoculated soil remained < log 1.7 cfu/g soil.

incubation. By day 30, NRRL 30797 was well established and remained present throughout the remaining incubation period. More rapid soil colonization was initially observed in samples containing single Pesta granules than single MB granules. The findings are likely due to the high content of farinaceous substance found within Pesta, which are known to promote rapid fungal growth (Connick et al., 1998). As pesta granules are completely disintegrated within 10 days of introduction to soil, no secondary reservoir inoculum is available compared to MB granules, which can maintain a prolonged availability of propagules. However, at the end of the incubation period (60 days), *A. flavus* populations were not

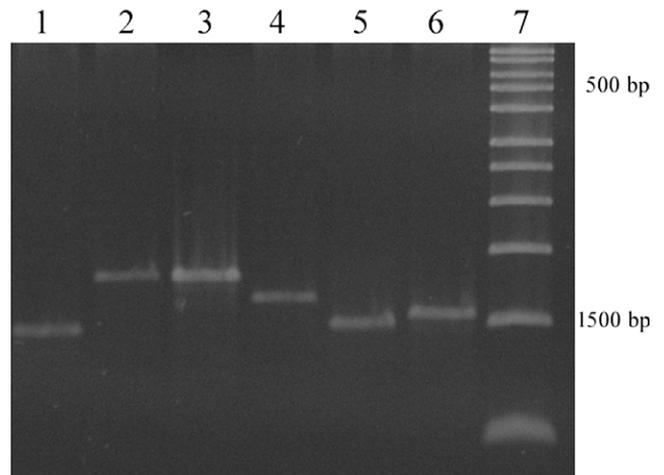
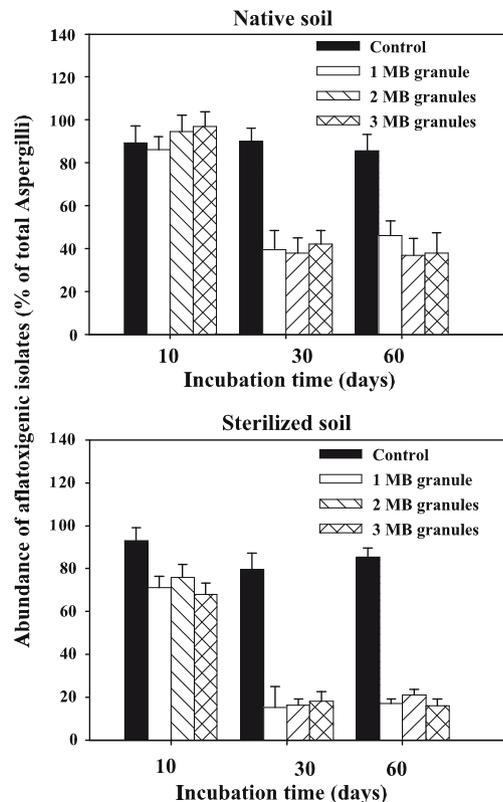


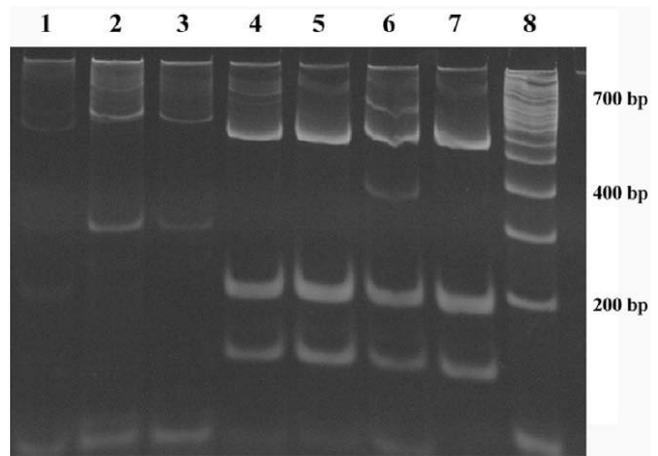
Fig. 7. Amplification profile of aflatoxin biosynthesis genes. Lane: 1 *aflD*, lane 2: *aflO*, lane 3: *aflP*, lane 4: *aflQ*, lane 5: *aflR*, lane 6: ITS (positive control), lane 7: 1 kb ladder.



**Fig. 8.** Ability of non-aflatoxigenic strain *A. flavus* NRRL 30797 colonized Mater-Bi® (MB) granules to competitively displace aflatoxin-producing strain *A. flavus* NRRL 30796 previously established in native or sterilized soil.

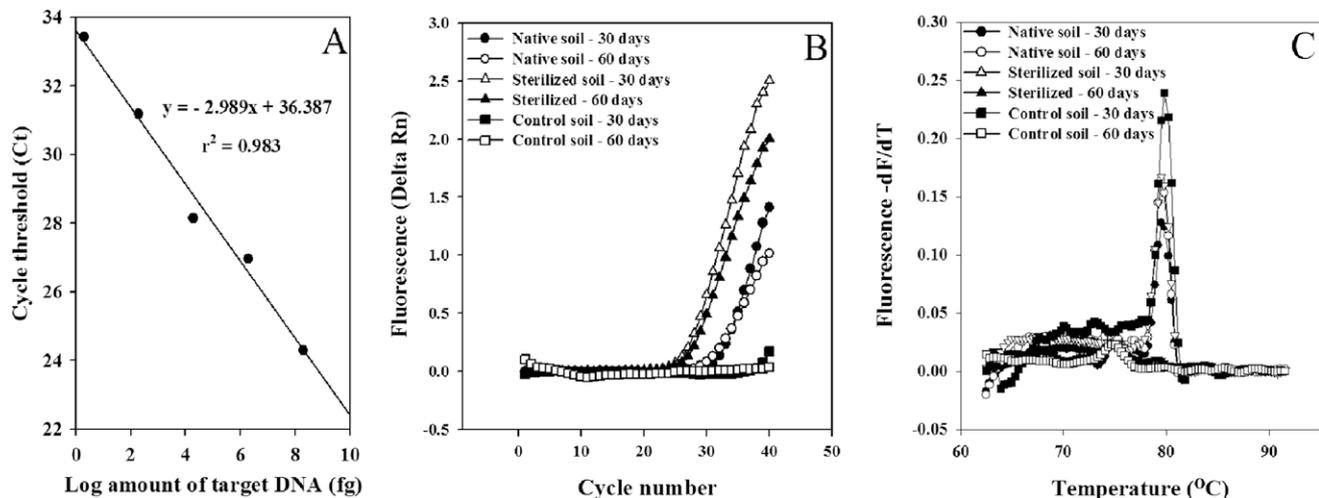
significantly different between Pesta and MB samples. It is apparent that the bioplastic matrix supports fungal growth (Fig. 5), but the resulting slow build up of *A. flavus* propagules may be due to the slower biodegradation rate in soil.

The relative abundance of non-aflatoxigenic isolates in soil samples inoculated with the toxigenic strain NRRL 30796 is reported in Fig. 8. Addition of MB granules containing the non-aflatoxigenic *A. flavus* strain NRRL 30797 resulted in decreased frequency of aflatoxigenic isolates over the 60-day incubation period, indicating a displacement of aflatoxigenic strain NRRL 30796. The greater abil-



**Fig. 9.** Ribosomal DNA internal spacer analysis of native and sterilized soil samples receiving increasing number of Mater-Bi® (MB) granules. Lanes 1, 2 and 3: samples of native soil containing 1, 2 and 3 MB granules, respectively. Lanes 4, 5 and 6: samples of sterilized soil receiving 1, 2 and 3 MB granules, respectively. Lane 7: *A. flavus* NRRL 30797, lane 8: 100 bp DNA ladder.

ity of the isolate NRRL 30797 to colonize and propagate in the soil in respect to the aflatoxin-producing strain NRRL 30796 has been previously demonstrated (Abbas et al., 2006). However, increasing the number of MB granules did not result in a decrease in aflatoxin-producing isolates. These findings are consistent with the results of the soil colonization experiment discussed above; increasing the number of propagules of the biocontrol strain was not followed by a significant increase of *Aspergilli* in soil. Similar results have been seen in work involving *Verticillium chlamydosporium* by Mauchline et al. (2002) who concluded that competition for nutrients between the introduced and natural occurring microorganisms is the major factor limiting growth of the biocontrol fungus. In addition to reduction of the toxigenic strain, RISA digested ITS-PCR products demonstrated that by 30-days of incubation the diversity of the total fungal community had decreased, resulting in a predominance of *A. flavus* NRRL 30797 (Fig. 9). Soil colonization by the biocontrol strain and reduction of the aflatoxigenic strain was significantly greater in sterilized soil than in native soil (Fig. 8). A similar rapid and intense colonization of sterilized soil by other introduced biocontrol fungi has been reported in the literature (Elad et al., 1981; Papavizas, 1985) and has been attributed



**Fig. 10.** Standard curve quantifying DNA of *A. flavus* NRRL 30797 (target DNA) obtained by qPCR with SyBR Green as the fluorescent dye. Ct values are averages of three independent dilution series (A). Representative qPCR analysis (B) and dissociation curves (C) of soil samples receiving single Mater-Bi® (MB) and incubated for 30 and 60 days.

**Table 3**

Total amount of *A. flavus* NRRL 30797 DNA isolated from soil samples containing the indicated number of Mater-Bi® (MB) or Pesta granules and incubated at 25 °C. Number are means of three replicates ± SE.

Incubation time/treatment	Amount of target DNA ( $\times 10^3$ fg g <sup>-1</sup> )		
	10 days	30 days	60 days
Soil + 1 MB granule	1.72 ± 0.31	20.12 ± 6.23	21.33 ± 3.99
Soil + 2 MB granules	0.90 ± 0.29	17.56 ± 4.91	19.57 ± 4.28
Soil + 3 MB granules	1.11 ± 0.35	19.30 ± 3.84	18.74 ± 2.98
Soil + 1 Pesta granule	25.32 ± 5.55	27.11 ± 6.01	24.96 ± 5.97

to the reduced inter-specific competition of fungal species in sterilized soil (Leandro et al., 2007).

The dynamics of the soil *A. flavus* population was also studied using qPCR (Fig. 10). Correlation between Ct values obtained from soil DNA and size of viable propagules, estimated by plate counting, gave a determination coefficient < 0.68 (data not shown). Low correlation can be due to a number of reasons. Efficiency of DNA recovery can depend on the initial total amount which is variable over time due to both cell growth and due to mycelia fragments that exist as multinucleate structures (Gow and Gadd, 1995). Additionally, qPCR does not discriminate DNA from viable and non-viable propagules (Mayer et al., 2003), thus artificially high representations of dead propagules may result. In the experiment described here, data are consequently expressed in terms of target DNA as calculated using the standard curve. As indicated in Table 3, the amount of target DNA increased during the first month of incubation and remained relatively constant during the remaining incubation period, which is compatible with result obtained using the cultural method (Fig. 6). In contrast to plant count data, amount of target DNA isolated from samples containing single Pesta granules was unaffected by incubation time. This was likely due to presence of the clay fraction of Pesta granules which would reduce the efficiency of DNA recovery. As expected, total *A. flavus* DNA isolated from MB granules introduced in soil increased over the incubation period, thus confirming that the proposed starch-based material supported fungal growth. Soil samples receiving increasing dosage of MB granules showed similar amount of target DNA as estimated by qPCR.

#### 4. Conclusions

Results of this laboratory study demonstrated that MB granules are an effective substrate for introducing a stable population of the biocontrol strain *A. flavus* NRRL 30797 in soil. The bioplastic product MB has a comparable efficiency for delivering Aspergilli propagules in the soil to the widely used formulations employing the wheat flour-based Pesta. The easy and rapid process proposed here for entrapping Aspergilli into MB granules, as well as the favorable physical characteristics of the granules, makes MB a practical option for delivering the non-aflatoxigenic *A. flavus* NRRL 30797 strain in the field. MB granules may also be adaptable for use in carrying other biocontrol fungi (i.e. *Trichoderma* spp. etc.). In conclusion, although, this preliminary data obtained under controlled conditions (i.e. soil moisture, air temperature, etc.) needs to be confirmed under field conditions, it appears that similar to the well established product Pesta, Mater-Bi technology should be adaptable to a number of other biocontrol agents. To our knowledge, this is the first paper suggesting the use of a bioplastic material for carrying biocontrol fungi.

#### Acknowledgements

We would like to thank the personnel of the Department of Agriculture of Novamont S.p.A. for providing the bioplastic mate-

rial and for technical assistance. A special thank to Ms. Bobbie Johnson and Ms. Lynn M. Libous-Bailey of the USDA-ARS for their scientific support. This paper was approved for publication as Journal Article No. J-11376 for the Mississippi Agricultural and Forestry Experiment Station, Mississippi State University.

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