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Induced selection of tebuconazole-resistant *Aspergillus flavus* isolates during germination of treated corn seeds

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ABSTRACT

Azole fungicides are used for spraying crops and also in seed treatments of corn, wheat and other important agricultural crops, in which seeds are dressed with a plastic-like coat containing an azole fungicide and other seed and seedling protection agents. In this study, the effect of tebuconazole in corn seed treatment on selecting for tebuconazole-resistant *A. flavus* isolates was investigated. Seed-borne *A. flavus* isolates growing during seed germination were tested for tebuconazole resistance. When seeds were treated with increasing dosages of tebuconazole, the relative abundance of resistant isolates increased. At the recommended dosage, up to 72.1 % of the seed borne *A. flavus* isolates that emerged from germinating seeds were resistant to tebuconazole. Resistance increased to 83.4 and 95.1 %, when dosages were doubled or quadrupled, respectively. Application of tebuconazole also increased the abundance of aflatoxin-producing isolates of *A. flavus*, from 32.2 % in untreated seeds to 67.4 % in seeds receiving the highest dosage. Results from this study suggest that seed treatment with tebuconazole should be included in the list of hotspots that induce resistance to azole antifungals and that measures and strategies, such as alternative fungicides with different metabolic targets, should be considered for reducing this risk.

1. Introduction

Seeds of numerous crop species are routinely treated with pesticides before planting to reduce the risk of pathogen and pest attacks during the vulnerable phases of seed germination and seedling standing [1,2]. Among the different techniques and approaches, with seeds of adequate size and uniform shape (i.e., corn, sunflower, cotton, etc.), pesticides are commonly applied by covering their surface with a thin plastic-like film coat containing one or two fungicides, an insecticide, and in most cases, a bird repellant [3]. Additives and dyes are then added to improve mechanical planting and to prevent adulteration of food and feed. In practice, film-coating is achieved by applying a small volume of liquid slurry to seeds which are rotating in a coating pan. Size and shape of film-coated seeds remain unaltered, with only a small increase in mass [4].

A major advantage of film-coating, and in general of seed treatment, with respect to broadcast and in-furrow applications, is that pesticides are precisely placed in strict proximity of the target of protection (i.e., germinating seeds and seedlings). For effective protection against pests and pathogens, slurries are prepared with a high dosage of pesticides, usually in the range of parts per thousand. Beside technical aspects, these elevated dosages could exert negative and/or selective pressure on microorganisms that come in contact with the artificial seed coat. This is especially the case of filamentous fungi, including soil- and seed borne fungi, and fungi that are located on the seed surface. Resistance development by fungal isolates that are exposed to field crop fungicides is becoming a serious issue, especially when these fungi are also of medical importance [5,6]. Many agricultural fungicides share the same metabolic target sites and have similar mode of action as medical antifungals [7]. An emerging issue regarding this phenomenon is the increasing occurrence of azole-resistant *Aspergillus fumigatus* and *A. flavus* isolates, two fungi that are responsible for invasive aspergillosis and are readily isolated from environmental samples, including soil and seeds [8,9]. Azoles are a group of antifungals that are widely used in medicine and agriculture. They were introduced in the 1970s and are currently the most used fungicides in crop protection for both foliar and seed

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treatment applications [10]. Tebuconazole is a widely used azole fungicide with an aromatic ring that gives it the potential for endocrine-disrupting effects. Despite intense research efforts aimed at developing novel anti-fungal agents, azoles remain the most prescribed antifungals for controlling fungal infections in humans, including invasive aspergillosis. While *A. fumigatus* is the most frequently reported agent causing aspergillosis infections, the incidence of *A. flavus* is increasing, especially in areas with hot and arid climates (e.g., Middle East, Asia and Africa) [11]. Other *Aspergillus* species are also involved in invasive aspergillosis, such as *A. terreus* and *A. nidulans* [12]. All these filamentous fungi are ubiquitous, living as saprophytes in soil, plant debris, compost and other organic-rich substrates, including seeds [8,13,14]. However, differently from the others, *A. flavus* is also an opportunistic plant pathogen, responsible for ear rot in corn and, most importantly, a major producer of aflatoxins which are potent carcinogenic toxins for humans [8]. *A. flavus* is commonly recovered from corn kernels, and when seeds are improperly stored (e.g., high seed moisture content), the fungus continues to grow and thus negatively impact seed quality in terms of both aflatoxin content and reduced germinability and vigor [15–17]. As stated above, film-coating corn seeds with fungicides serves to prevent the growth of seed-borne fungi than can reduce seedling germination and growth, and for protecting the seeds from being infected by soil-inhabiting pathogenic fungi [2]. Consequently, applications of seed film coats containing one or more fungicides could also provide conditions for increasing the frequency of antifungal agent-resistant isolates within the populations of seed-borne fungi and soil-inhabiting fungi living in proximity to the artificial coat. Recent studies have investigated the role of soil fungicidal residues from foliar applications in promoting development of antifungal agent-resistance, focusing on *A. fumigatus* and azole fungicides. However, a limited number of studies have investigated *A. flavus* and none of these have specifically dealt with *A. flavus* and azoles when applied as a seed treatment [10,18].

The main objective of the present study was to evaluate the hypothesis that coating corn seeds with tebuconazole, an azole fungicide widely used for treatment of multiple seed species, could induce the selection of tebuconazole-resistant seed-borne *A. flavus* during the seed germination process. The study also focused on the effect of tebuconazole seed treatment on the relative abundance of aflatoxigenic and non-aflatoxigenic *A. flavus* isolates recovered from germinating seeds.

2. Materials and methods

2.1. Seeds and seed film-coating

Seeds of the commercial corn hybrid Kristal (KWS Italia S. p.A., Forlì, Italy) were surface sterilized by washing for 3 min in a 1 % NaClO solution, followed by rinsing four times in ultrapure sterile water. Surface-sterilized seeds were then film coated with an aqueous slurry prepared with the commercial formulation Sepiret® 9290 (Basf Corp., Ludwigshafen, Germany) with the addition of tebuconazole, 1-(4-chlorophenyl)-4,4-dimethyl-3-(1H-1,2,4-triazol-1-ylmethyl)pentan-3-ol (Pestanal®, analytical standard; Merck KGaA, Darmstadt, Germany) at the following dosages: 5, 10, and 20 mg mL^{-1}, corresponding to 17 (1x), 34 (2x), and 68 (4x) μg $seed^{-1}$. Before application, the slurry was sterilized by passing through a 0.2 μm sterile filter. Seeds (50 g) were then transferred into a sterile 50-mL centrifuge tube and coated by adding 0.25 mL of slurry while vortexing the tubes at maximum speed for 2 min using a Vortex- Genie 2 (Scientific Industries Inc., Bohemia, NY, USA). Visual inspection confirmed that seeds were completely coated by the colored coating material. The procedure was repeated using a bio-based coat formulation containing extruded starch (5.5 % w/v), Arabic gum from acacia tree (2.7 % w/v), soybean lecithin (0.3 % w/v) and soy wax (0.05 % w/v), as described in Accinelli et al. [3].

2.2. Recovery of seed-borne microorganisms from germinating seeds

Corn seeds were incubated in single seed germination tubes as shown in Fig. 1. Seed-borne microorganisms were recovered from germinating corn seeds using germination tubes (Fig. 1) specifically designed to avoid microbial contamination and to exclude any potential effects from external nutrient sources (i.e., agar-based germination substates) [19]. Briefly, seeds were placed in the center of a 2-cm diameter plastic support, which was provided with two lateral sterile cotton filters (diameter of 10 mm; length of 18 mm) that served for keeping the seed moistened when they were wetted. The disc was placed between two conical 50-mL centrifuge tubes, connected by a screw cylinder. Except for the centrifuge tubes, all other components were manufactured using a Form 3 L SLA 3D Printer equipped with a 100 μm resolution clear resin. Printer machine and resins were obtained from Formlabs Inc. (Boston, MA, USA). The two filters were moistened with a fixed volume of sterile ultrapure water (1.5 mL each filter plug) and then tubes were incubated in a germination chamber at 25 °C with 12 h of light per day.

Germination percentage was recorded daily with mean germination time (MGT) calculated as follows: $MGT = (\Sigma n_i \bullet h_i)/\Sigma n_i$, in which n_i is the number of seedlings present on interval i, and h_i is the number of hours since the beginning of the test [20]. Seeds (100 per treatment) were considered to have germinated after the radicle emerged. After 4 days from the beginning of seed incubation, cotton plugs were aseptically removed and directly used for microbial evaluation.

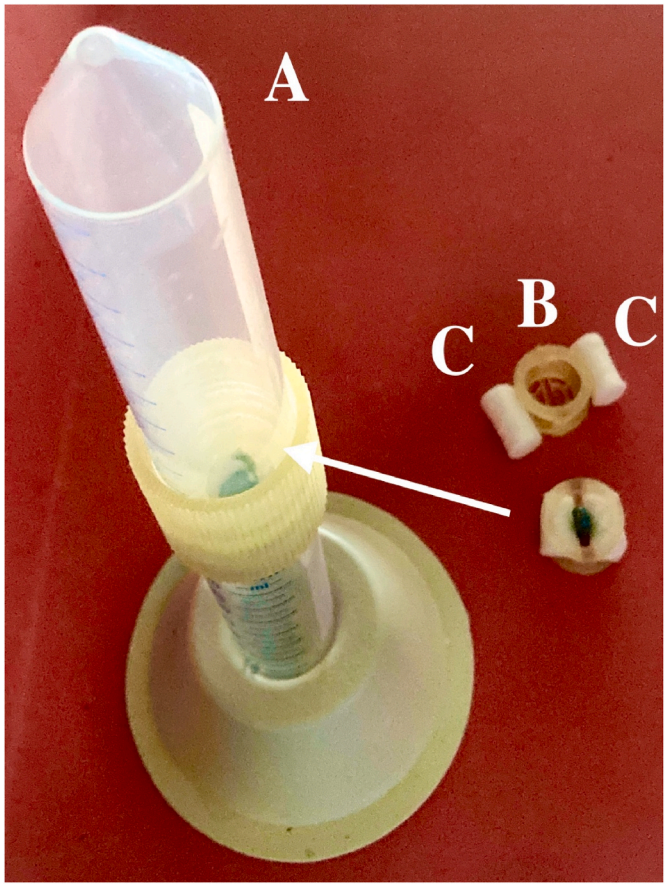

Fig. 1. Seeds were incubated in single-seed germination tubes (A), consisting of two connected centrifuge tubes containing a plastic support (B) provided with two cotton plugs (C) for both moistening the seeds and entrapping seed-borne microbes.

2.3. *Aspergillus flavus* isolation and qPCR quantification, and composition of the fungal community

Cotton plugs recovered from germination tubes were transferred to 15-mL centrifuge tubes containing 19 mL of autoclaved 0.02 % Tween 20 solution and shaken on a horizontal shaker for 30 min at room temperature. Aliquots of 100 μL were spread onto plates of modified 2,6-dichloro-4-nitroaniline Rose Bengal (MDRB) agar medium and incubated at 37 °C for 7 days. Isolates were randomly selected, sub-cultured on PDA and then tested for azole resistance and aflatoxin production. For both analysis a total of 50 randomly selected *A. flavus* isolates were used.

Cotton plugs from incubated samples were processed for quantifying *A. flavus* using a molecular approach. Specifically, quantification was performed by qPCR following the procedure described in Accinelli et al. [21]. The total DNA from 100 aliquots of the above-mentioned microbial dispersion was isolated using the commercial kit DNeasy® UltraClean® Microbial Kit (Qiagen Corp, Hilden, Germany) following the manufacturer's instructions. DNA was quantified using a BioDrop spectrophotometer (BioDrop Ltd, Cambridge, UK) and then amplified with an Open qPCR (ChaiBio, Santa Clara, CA, USA) using the following conditions: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Each 25 μL of reaction mixture contained 12.5 μL of 2 × TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 0.2 μM of each primer [21], and 40 ng of DNA. Samples were amplified on a standard curve (r^2 = 0.92; efficiency = 94 %; slope = - 0.21) generated by plotting cycle threshold values (Ct) against known spore concentration values.

DNA samples that were recovered from cotton plugs were then used for next-generation sequencing (NGS) analysis of the seed-borne fungal community. DNA samples were sequenced by BMR Genomics S. r.l. (Padova, Italy) with Illumina high-throughput sequencing methodology. The fungal ribosomal ITS2 region was amplified with ITS3_KYO2 (GATGAAGAACGYAGYRAA) and ITS4r (TCCTCCGCTTATTGATATGC) primers. A total of 1,481,944 paired-end reads were produced by the amplification, with an average of 82,330 paired-end reads per sample. Microbiome bioinformatics were performed using an adapted Snakemake pipeline [22,23]. Raw sequence data were quality checked with FastQC. Sequence quality was improved using the Trimmomatic tool [24] by clipping Illumina adapters, removing bases with a Phred quality score lower than 15, and, sequences shorter than 36 bp. OTUs clustering was performed with DADA2 [25], such as the construction of their relative frequency table. Taxonomy was assigned with QIIME 2 2017.4 (Bolyen et al., 2019) using the q2-feature-classifier [26] classify-sklearn naïve Bayes taxonomy classifier against the UNITE [27] reference sequences database (97 % similarity). Sequences were deposited in the European Nucleotide Archive (ENA) at EMBL-EBI (accession number PRJEB72729).

2.4. Tebuconazole susceptibility and aflatoxin production of recovered *A. flavus* isolates

Isolates of *A. flavus* that were recovered from germinating seeds were evaluated for their susceptibility to the fungicide tebuconazole and their ability to produce aflatoxins.

Tebuconazole susceptibility test was performed following the microbroth dilution method outlined by the Committee for Antimicrobial Susceptibility Testing (CAST), reference method version 9.3.1 [28]. Fungal isolates were sub-cultured on potato dextrose agar (PDA) and spores were collected as described above. Spore dispersions were adjusted to 3 x 10^5 spores mL^{-1} and aliquots of 100 μL were used to inoculate an equivalent volume of 2 x RPMI 1640 medium with 2 % glucose (ThermoFisher Scientific Inc., Waltham, MA, USA), contained in each single well of 96-well microplates. Tebuconazole solutions (30 μL) were added to the wells to achieve final concentrations of 0.03, 8 and 16 mg L^{-1}. Control wells prepared with no tebuconazole and uninoculated wells were included. Two *A. flavus* isolates, NRRL 30796 and NRRL 30797, were included as quality control. After incubation for 48 h at 37 °C, wells were visually inspected and the minimal inhibitory concentration (MIC) of tebuconazole was determined as the lowest concentration of the antifungal that completely inhibited fungal growth. Isolates with MIC above 16 mg L^{-1} were considered resistant to the fungicide tebuconazole. Analytical grade (>98 % purity) tebuconazole was purchased from Merck KGaA (Darmstadt, Germany).

For assessing the capability of *A. flavus* isolates to produce aflatoxin B1, plugs from PDA plates with active fungal growth were transferred to test tubes containing 5 mL of yeast extract sucrose broth and incubated without shaking for 7 days in the dark at 30 °C. Samples were then extracted with chloroform (2 mL) by shacking for 1 min and extracts evaporated to dryness in vacuo. Residues were dissolved in methanol/H_2O (70:30, v:v) and aflatoxins concentration determined by HPLC, following the method of Accinelli et al. [29]. A calibration curve was obtained using solutions with known concentration of analytical grade aflatoxin B1 (purity ≥98.0 %; Merck KGaA, Darmstadt, Germany). The limit of detection was 0.1 ng g^{-1}. Dry weight of mycelial mats was determined after air drying for 48 h at 70 °C.

2.5. Statistical analysis

Experimental data were processed by one-way analysis of variance ANOVA, using the software SPSS ver. 29 (SPSS Inc., Chicago, IL, USA). Means were compared by Fisher's least significant difference (LSD), and P values < 0.05 were considered statistically significant.

3. Results and discussion

3.1. Recovery of seed-borne *A. flavus* isolates from germinating seeds

Seed germination data are summarized in Table 1. Application of the commercial or the bio-based formulations to non-sterile or surface-disinfected seeds did not affect the germination percent or the mean germination time. These two parameters were also unaffected when the two film-coat slurries were applied with the addition of tebuconazole. Although, under some circumstances (e.g., chilling conditions), tebuconazole can affect corn seed germination, in general, when applied at recommended doses, germination and seedling growth are not influenced by this fungicide [30].

As summarized in Table 1, *A. flavus* was detected in 57.4 % of the

Table 1
Germination, mean germination time and *A. flavus* contamination of corn seeds. The following treatment of corn seeds were studied: uncoated and non-disinfected seeds; uncoated surface disinfected seeds; and surface disinfected seeds that were then coated with commercial or bio-based formulations containing the fungicide tebuconazole at 0, 1, 2, and 4 times the recommended dosage. Data are presented as mean ± standard deviation. Values followed by the same letter in the same column are not significantly different (P > 0.05).

Seeds	Germination	Mean germination time	*A. flavus*-contamination
	(%)	(h)	(%)
Uncoated	99.9 ± 1.0 a	49.0 ± 2.1 a	87.3 ± 5.1 a
Uncoated/ Disinfected	98.9 ± 1.1 a	48.5 ± 1.7 a	57.4 ± 2.4 b
Commercial Coat			
0x	99.7 ± 1.1 a	44.2 ± 1.1 a	51.2 ± 3.1 c
1x	100.0 ± 1.3 a	50.9 ± 1.8 a	12.4 ± 2.7 d
2x	97.8 ± 1.4 a	44.0 ± 1.1 a	8.1 ± 2.2 e
4x	99.8 ± 1.1 a	44.5 ± 1.3 a	3.3 ± 1.4 f
Bio-Based Coat			
0x	99.3 ± 0.9 a	44.0 ± 1.1 a	56.0 ± 3.5 b
1x	99.9 ± 1.3 a	43.7 ± 1.3 a	25.2 ± 2.2 g
2x	98.8 ± 1.7 a	44.2 ± 1.0 a	17.3 ± 3.1 d
4x	99.5 ± 1.0 a	43.9 ± 1.1 a	15.1 ± 2.9 f

untreated and surface-disinfected germinating seeds, confirming that this fungus is a common colonizer of corn kernels and not only one of the numerous soil-borne microorganisms that remain on the seed surface after being transported by wind, insects or other vectors [8,31]. As expected, the percentage of *A. flavus*-contaminated seeds was significantly higher in germinating seeds that were not surface-disinfected, with values of contaminated samples up to 87.3 %. Fewer *A. flavus* isolates were recovered from germinating seeds that were coated with the commercial formulation, while the bio-based formulation did not affect the number of isolates.

Seeds are infected by various microorganisms, including pathogen and endophytic species [32,33]. However, and for different reasons (e.g., competition among species, diversity in terms of growth condition requirements), some seed-infecting microorganisms can grow and proliferate during seed germination and seedling elongation while others do not [13]. A common approach used to investigate seed-borne microorganisms is based on recovering microbial propagules or microbial DNA from seeds after crushing the entire seeds. In other methods, seeds are incubated on agar-based nutrient media and then growing microorganisms are picked using inoculation loops or pipette tips [34,35]. While DNA-based approaches do not effectively discriminate among seed-borne microorganisms that are either actively growing on germinating seeds, dead or not growing, cultural methods are limited to culturable microorganisms [36]. These investigations are even more complicated when seeds are treated with pesticides. In the case of corn, and as described above, seed treatment is achieved by covering seeds with a self-adhering plastic-like coat containing formulants (e.g., binders, fillers, dyes, etc.) and pesticides (e.g., fungicides, insecticides, bird repellents, etc.). When seed treatment is prepared with one or more fungicidal active ingredients, the artificial coat surrounding the seed is expected to act as a shield against unwanted fungi coming from the soil, trying to pass through and infect the seeds. While this practical aspect has been described in the technical and scientific literature (i.e., effectiveness of seed treatment to control fungal infestation), the effects of fungicidal seed treatment on selection and fitness of seed-borne fungi have remained surprisingly unexplored [3,29].

When seeds were film-coated using the commercial polymeric coat, the estimated number of *A. flavus* propagules that were recovered from the two cotton plugs showed a 10.8 % reduction with respect to uncoated seeds (Table 1). This was not observed with the bio-based coating. Application of the synthetic polymer-based formulation likely resulted in the formation of a more compact and less penetrable layer than that obtained with the bio-based slurry. More specifically, at the end of the 4-day incubation period, no visible cracks or fissures were observed in the synthetic coat. In contrast, the bio-based coat began to lose consistency, with visible small fissures. In the present experiment, this rapidly degradable coat was specifically included to compare with the more persistent and firmly adherent synthetic coat.

As expected, treating the seeds with the commercial formulation containing tebuconazole at the recommended dose (17 μg active ingredient seed^{-1}) resulted in a significant reduction in the number of recovered *A. flavus* propagules from germinating seeds. More specifically, the number of propagules decreased to 78.4 %. A further decrease was observed when tebuconazole was applied at the 2x dosage. Only a reduced number of *A. flavus* isolates were capable of crossing the tebuconazole-containing coat when the antifungal dosage was quadrupled. Comparable results were observed when the synthetic polymeric coat was replaced with a bio-based and less persistent coat, except that the effect of tebuconazole was less pronounced. For instance, at the 1x, 2x and 4x dosage, the percent of recovered *A. flavus* propagules were of 25.2, 17.3, and 15.1 %, respectively (Table 1). As discussed above, this was likely due to the more porous and fissured structure of the bio-based coat. The use of the qPCR approach was chosen for estimating the potentiality of tebuconazole-treated coat to select *A. flavus* isolate capable to cross this selective barrier. Coupled to the tebuconazole susceptibility test, this provide information on the extend of this phenomena.

Various seed-borne fungal species were recovered during seed germination using next-generation sequencing (NGS) techniques (EMBL-EBI accession number PRJEB72729; Table 2). The dominant fungal class was *Mucoromycetes*, followed by *Dothideomycetes*, and *Sordariomycetes*. *Rhizopus arrhizus* was the most abundant species, followed by *Alternaria alternata*, and *A. flavus*. These findings are consistent with those discussed above, thus confirming that *A. flavus* is readily isolated from corn kernels. While *A. flavus* was detected in control and treated samples, *A. neoniger* was detected in treated samples, but not in the untreated control. *A. sydowii* was only detected (<0.01 %) in samples receiving the 2x tebuconazole dosage. Application of tebuconazole at the dosage of 2x and 4x, increased the relative abundance of *A. flavus*. These differences are likely due to the selective effect of the fungicide tebuconazole and the occurrence of seed-borne fungal isolate that have acquired tolerance to this fungicide.

Even at elevated tebuconazole doses, the artificial coat surrounding seeds was only partially effective in inhibiting the growth of seed-borne fungi, presumable because the fungicidal barrier was not impenetrable. Importantly, observed differences in the composition of the fungal community between untreated and tebuconazole-treated seeds indicated that when applied as a seed treatment, tebuconazole affects the number and species of seed-borne fungi that can be dispersed from the seeds into the soil and the environment.

3.2. Susceptibility to tebuconazole and aflatoxigenicity of recovered *A. flavus* isolates

Seed-borne *A. flavus* isolates that were recovered from germinating seeds were evaluated for their susceptibility to tebuconazole using the CAST protocol and their potential to produce aflatoxins. As summarized in Fig. 2, up to 7.1 % of those recovered from untreated seeds showed resistance to the fungicide tebuconazole, thus confirming that resistance to this class of chemicals is widely spread in environmental *A. flavus* isolates [37,38]. While most of the studies concerning resistance of aspergillosis-causing fungi to azole fungicides have focused on the primary agent of invasive aspergillosis, *A. fumigatus*, only limited information is available for the closely related fungus, *A. flavus*. Recent investigations have indicated a major role for agricultural applications of azole fungicides in the development of selective resistance in these two species [39,40].

Being an opportunistic plant pathogen infecting oil-rich seeds, such as peanuts, cottonseeds, corn kernels, and others, seeds can easily spread spores and other propagules of environmental *A. flavus* isolates, including azole fungicide-resistant isolates. Since seeds of these species are routinely treated with fungicides, including azole fungicides, the potential effect of tebuconazole, an azole fungicide widely used in seed treatment, on selecting resistant seed-borne *A. flavus* isolates was evaluated in this study. As discussed above, application of tebuconazole at the recommended dosage blocked and/or deactivated most of the seed-borne *A. flavus* isolates (Table 1). However, when seeds were coated with a commercial polymer slurry containing tebuconazole, the percentage of resistant isolates increased to 72.1 %. This percentage further increased when the tebuconazole dosage was doubled. At the 4x dosage, 95.1 % of the recovered isolates were resistant to tebuconazole. When the commercial polymer slurry was replaced with a bio-based and rapidly degradable but more permeable coating, this phenomenon was less pronounced. More specifically, with the 1x, 2x, and 4x tebuconazole dosage, the percent of resistant isolates was 58.1, 70.0, and 68.9 %, respectively (Fig. 2). This may have been due to some isolates growing through fissures and pores in the tebuconazole-containing bio-based coating, which was not observed with the commercial coat.

In addition to soil, particularly the organic debris in it, other hotspots for fungicide resistance development in *A. fumigatus* that have been mentioned include wastes from azole fungicide-treated vegetative propagules (e.g., flower bulbs), industrial and home composting heaps, and treated seeds [5,6]. Surprisingly, considering the importance of the

Table 2
Relative frequency of more representative fungal classes and species detected from germinating seeds using the next-generation sequencing (NGS) approach. Seeds were surface-disinfected then coated with a commercial formulation containing tebuconazole at the recommended dosage of the fungicide, and at multiples of that dosage.

Class *Species*	Frequency (%)					
	Uncoated	Uncoated/disinfected	0x	1x	2x	4x
Mucoromycetes	–	92.03	65.12	56.98	92.08	78.88
Rhizopus arrhizus	–	92.03	65.12	55	92.08	78.88
Dothideomycetes	86.67	2.75	18.04	26.77	4.87	11.60
Alternaria alternata	25.56	2.30	9.56	21.80	1.29	9.32
Sordariomycetes	–	3.45	10.13	2.95	<1	6.51
Eurotiomycetes	–	<1	3.02	10.94	<1	1.65
Aspergillus flavus	–	<1	2.18	10.58	<1	1.56
Aspergillus neoniger	–	–	<1	<1	<1	<1
Aspergillus sydowii	–	–	–	–	<1	–

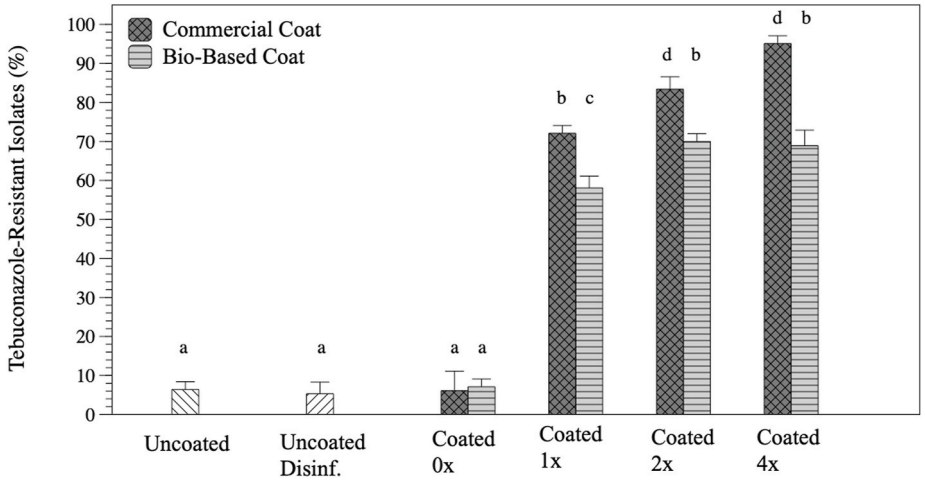

Fig. 2. Percentage of seed-borne *A. flavus* isolates showing resistance to the fungicide tebuconazole. Isolates were recovered from germinating seeds that were coated with commercial or bio-based formulations containing the fungicide tebuconazole at 0, 1, 2, and 4 times the recommended dosage of 17 μg seed^{-1}. Before coating, seeds were surface disinfected. Data were calculated with respect to the total recovered seed-borne *A. flavus* isolates. Each bar is presented as mean ± STD. Bars with the same letters are not significantly different (P > 0.05).

application of azole fungicides in the treatment of crop seeds, none of the reported studies have specifically focused on the role of treated seeds on the selection of azole fungicide resistance in the genus *A. flavus*.

Application of tebuconazole as a seed treatment also affected the ratio of aflatoxigenic to non-aflatoxigenic isolates (Fig. 3). In untreated germinating seeds, 32.2 % of *A. flavus* isolates were able to complete

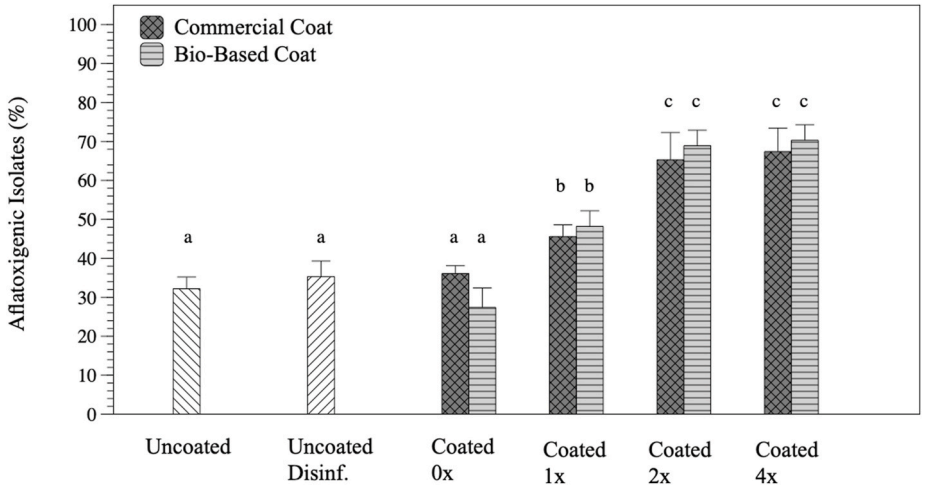

Fig. 3. Percentage of seed-borne *A. flavus* isolates able to produce aflatoxins. Isolates were recovered from germinating seeds that were coated with commercial or bio-based formulations containing the fungicide tebuconazole at 0, 1, 2, and 4 times the recommended dosage (17 μg seed^{-1}). Before coating, seeds were surface disinfected. Data were calculated with respect to the total recovered seed-borne *A. flavus* isolates. Each bar is presented as mean ± STD. Bars with the same letters are not significantly different (P > 0.05).

aflatoxin B1 biosynthesis and surface disinfection had little but not significant effect for seeds coated with commercial or bio-based formulations with no added tebuconazole. In contrast, when tebuconazole was incorporated into the coat at the suggested dosage (1x dosage), 45.6 % of isolates produced aflatoxin B1, and the percentage increased to 65.3 and 67.4 % when the dose was doubled and quadrupled (2x and 4x dosages), respectively. No significant differences were observed between commercial and bio-based formulations.

Recent investigations have demonstrated that the capability of *A. flavus* isolates to produce aflatoxins results in a fitness advantage, especially when the fungus competes with other organisms, such as bacteria and insects, for food resources in nutrient-rich substates, as corn kernels [41]. Although the ecological role of a large number of toxins and other secondary metabolites is unclear, in some circumstances, toxins such as aflatoxins, may play a role in competition among microbial species by alteration of nutrient sources [42]. It is not clear how an effective fungicide such as tebuconazole could reduce the nutrient value of corn seeds to seed-borne fungi, including *A. flavus*, thus giving an advantage to aflatoxin-producing isolates. An alternate explanation is that the characteristics of microbes that enable them to acquire mycotoxin biosynthetic cassette genes also enable the acquisition of genetic elements that confer azole fungicide resistance. It has been proposed that mycotoxin and antibiotic biosynthetic cassette genes are acquired using genome mining capabilities possessed by certain types of microbes found in soil [43]. These genome mining capabilities are presumed to be based on several better understood processes such as natural competence and transposons. If this proposal is true, when tebuconazole was used to select for fungi on corn seeds that were capable of acquiring azole fungicide resistance genes from their environment, it was in practice selecting for microbes with functional genome mining capability and aflatoxin-producing *A. flavus* isolates would be expected to be among the types of microbes selected for.

4. Conclusion

This series of experiments using specifically designed single seed test tubes indicated that application of the agricultural fungicide tebuconazole as a seed treatment should be included in the list of hotspots that select for *A. flavus* isolates resistant to the fungicide. In addition, the ratio of aflatoxigenic to non-aflatoxigenic *A. flavus* isolates also increased in tebuconazole-treated seeds. Considering the importance of azole fungicide resistance in *Aspergillus* species, and the widespread use of azole-class fungicides in agriculture, the effects of fungicide-containing seed treatments of agricultural crops on driving antifungal resistance should be included in the evaluation of these risks, and more studies are necessary to better elucidate this aspect.

CRediT authorship contribution statement

Chiara Morena: Software, Investigation, Conceptualization. **Cesare Accinelli:** Writing – original draft, Conceptualization. **Veronica Bruno:** Writing – review & editing, Resources. **Hamed K. Abbas:** Supervision. **Ryan T. Paulk:** Validation. **W. Thomas Shier:** Writing – review & editing.

Research data

Sequences were deposited in the European Nucleotide Archive (ENA) at EMBL-EBI (accession number PRJEB72729), other data are available from the corresponding author upon reasonable request.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Abbreviations:

MGT –: Mean Germination Time
MDRB –: Modified 2,6-dichloro-4-nitroaniline Rose Bengal
PDA –: Potato Dextrose Agar
Ct -: Cycle threshold
NGS -: Next-generation Sequencing
OTUs -: Operational Taxonomic Unit
MIC -: Minimal Inhibitory Concentration
HPLC –: High Performance Liquid Chromatography
LSD -: Fisher's Least Significant Difference