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RESEARCH

Application of antifungal metabolites from *Streptomyces philanthi* RL-1-178 for maize grain coating formulations and their efficacy as biofungicide during storage

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Abstract

The major safety risk of maize grain is contamination with mycotoxins. In this study, a maize-coating formulation containing freeze-dried culture filtrate of *Streptomyces philanthi* RL-1-178 (DCF RL-1-178) was developed and evaluated to prevent the growth of mycotoxins during maize grain storage. *In vitro* studies using confrontation tests on PDA plates indicated that *S. philanthi* RL-1-178 inhibited the growth of *Aspergillus parasiticus* TISTR 3276 (89.0%) and *A. flavus* PSRDC-4 (95.0%). The maize grain coating formulations containing the DCF RL-1-178 (0, 5, 10, and 15% (v/v)) and the polymer polyvinylpyrrolidone (PVP-K90, 4.0% (w/v)) were tested for their efficacy in *In vitro* and during 5 months storage. In *In vitro* assay, maize coating formular containing the optimum concentration (15.0%, v/v) of the DCF RL-1-178 exhibited 54.80% and 54.17% inhibition on the growth of *A. parasiticus* TISTR 3276 and *A. flavus* PSRDC-4 respectively. The inhibition was also illustrated by the microstructures of interactions between the coated maize grains with or without the DCF RL-1-178 and the fungal pathogens observed under microscope and SEM. Incorporating the DCF RL-1-178 or fungicidal Metalaxyl® into the polymer PVP-K90 maize grains coating resulted in the complete inhibition of the production of aflatoxin B_1 (analysed by HPLC) by the two aflatoxigenic pathogens after 5 months storage at room temperature. However, the shelf-life was shortened to only 3 months during storage at room temperature with 90% relative humidity. Overall, the application of the 10–15% DCF RL-1-178 into the maize grain coating formular provides a new alternative measure to control the mycotoxins during storage for at least 5 months. The *In vitro* cell cytotoxicity study showed that a concentration of 15% (v/v) or 1000 µg/mL of the DCF RL-1-178 had a strong cytotoxic effect on Vero cells. These findings indicate that DCF RL-1-178 is a potential biofungicide for controlling mycotoxins contamination in maize seed storage for planting, but not maize grain storage for animal feed.

Keywords Grain coating formular · Metabolites from *Streptomyces philanthi* · Biofungicide · Aflatoxigenic pathogen · Maize grain storage

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Introduction

Maize (*Zea mays* L.) is a widely cultivated cereal used as a source of food, forage and processed products for industry. Aflatoxin contamination is a prevalent issue in tropical countries, such as Thailand, due to the warm and humid climate. To ensure safety, strict regulations are in place globally to control the levels of aflatoxins in food and animal feed. Additionally, cereals also make a significant proportion of animal feed in all parts of the world (Alvarado et al. 2017). Aflatoxin contamination in cereals and cereal-based products is a prevalent concern due to fungal spoilage and mycotoxins. The risk of contamination increases during growing, harvesting, storage, transport and processing stages as the fungal infection can lead to mycotoxin contamination (Chulze 2010). *Aspergillus flavus* and *Aspergillus parasiticus* are prevalent aflatoxigenic pathogens that can infect a wide range of crops, both pre- and post-harvest, including maize, peanut, rice, cottonseed, and their products (Gong et al. 2019). Infections of maize with two aflatoxigenic fungi can cause major seriousness in grains during storage (Ng'ang'a et al. 2016; Logrieco et al. 2021). They can produce highly toxic and carcinogenic aflatoxins, posing a major threat to animal and human health worldwide (Bailly et al. 2018; Khan et al. 2021). The potential for humans and animals to be exposed to aflatoxins from contaminated maize is a major food safety issue, especially in regions where maize is a staple food and conditions are favorable for fungal growth and aflatoxin production. There are multiple reports documenting aflatoxin contamination of maize and maize-based products from almost all parts of the world (Lee and Ryu 2017). To curb the aflatoxins contamination in food crops, countries have imposed different laws regarding the level of these toxins in food crops. The United States Food and Drug Administration (USFDA) has established strict regulations for aflatoxin levels, with a limit of 20 ppb (parts per billion) in food and feed products, and 0.5 ppb in milk products. The European Union (EU) also has regulations in place for aflatoxin levels in food products, with limits ranging from 2 to 4 ppb (Gurtler and Keller 2019).

Management of plant diseases is important for most crops, and it is particularly critical for the production of high-quality grains. Treatment of maize is an effective way to eradicate or reduce seed-borne pathogens, especially when producing high-quality maize grain with a lower percentage of fungal infection is the goal (Mancini and Romanazzi 2014). In the past, maize treatments were carried out mainly by applying fungicides, and even now this remains the most effective means. However, as organic farming gains popularity, alternative methods that exclude the use of fungicides are becoming increasingly necessary. Developing new and efficient alternatives for crop protection, particularly for widely cultivated crops such as maize, is important. Biological seed treatments can be a viable alternative to chemical control of many seed-borne and food spoilage pathogens (Accinelli et al. 2018; Weaver and Abbas 2019; Rivas-Franco et al. 2020).

Film-coating is a technique that involves treating seeds with an aqueous solution containing a polymer or a mixture of polymers along with fungicide active ingredients to protect the seed from fungal pathogens. The process results in the formation of a thin layer covering the seeds (Accinelli et al. 2018). Film-coating does not alter the shape or seed size, but improves their flowability and reduces fungicide dust-off by the addition of sticking agents (Accinelli et al. 2016a, 2018). In recent years, the desire for more environmentally-friendly pest control methods has led to increased research on effective microbial biocontrol agents and formulations as an alternative or supplement to synthetic pesticides, for both field broadcast and seed treatment applications (Mancini and Romanazzi 2014; Accinelli et al. 2016a, b, 2018).

Among these techniques, seed coating is one of the most promising techniques for improving seed quality, vigor and field performance, by using appropriate coating agents at suitable concentrations (Rocha et al. 2019). Coating seeds with desirable agents such as chemicals (e.g. fungicides) or specific microbial species (e.g. bacteria, fungi, actinomycetes) or a combination of multiple materials has the potential to enhance chemical delivery and/or microbial colonization in a cost-effective way at field scale (Ma et al. 2019; Li et al. 2021).

Bacterial strains of the genus *Streptomyces* have been the subject of extensive study due to their potential use as biocontrol agents (Li et al. 2021; Ngalimat et al. 2021; Carlucci et al. 2022; Fu et al. 2022; LeBlanc 2022; Le et al. 2022). *Streptomyces* are known to produce a variety of molecules with potential biocontrol properties, such as antibiotics (Igarashi et al. 2005; Quinn et al. 2020), antifungal compounds (Boukaew et al. 2014, 2017, 2020a,b, 2021; Chen et al. 2016; Li et al. 2011; Shakeel et al. 2016), and hydrolytic enzymes (glucanase, chitinase) (Prapagdee et al. 2008; Boukaew et al. 2016; VazJauri et al. 2016), which give them the ability to inhibit fungal growth. The use of antifungal metabolites produced by antagonistic microorganisms for seed coating is a limited field of study and application.

The objective of this study was therefore to develop and evaluate a film-coating formulation containing antifungal metabolites of *S. philanthi* RL-1-178 to prevent the growth and aflatoxin production of *A. parasiticus* and *A. flavus* during maize grain for long-term storage by means of a novel maize grain treatment technology. Additionally, the cytotoxicity of antifungal metabolites has also been discussed.

Materials and methods

Microorganisms and preparation of freeze-dried culture filtrate

The strain *Streptomyces philanthi* RL-1-178 was isolated from the rhizosphere of chili pepper in southern Thailand (Boukaew et al. 2011). To prepare freeze-dried culture filtrate, submerged cultures of *S. philanthi* RL-1-178 were grown at 30 ℃ on a rotary shaker at 150 rpm in a 250 mL flask containing 100 mL glucose yeast-malt extract medium (GYM) with the pH adjusted to 7.0 using 5 M NaOH before autoclaving. After 3 days, 10% (v/v) aliquots of the seed culture was inoculated into a 5 l bioreactor (New Brunswick™ BioFlo® 415 Sterilize-in-Place (SIP) Fermentor, Eppendorf North America) containing 4 l tuna condensate medium (pH was adjusted to 7.0 before autoclaving) and after ten more days of incubation under the same conditions, the culture broth was centrifuged (8880 × *g* for 20 min) to obtain a cell-free supernatant and a pellet. The cell-free supernatant was passed through a 0.45 µm pore size filter to exclude bacteria. After that, the cell-free culture filtrate was freeze-dried by vacuum freeze-dryer at the Office of Scientific Instrument and Testing, Prince of Songkla University (PSU) to obtain the freeze-dried culture filtrate from *S. philanthi* RL-1-178 (so-called DCF RL-1-178).

Aflatoxin-producing fungi and spore inoculum preparation

The fungal strains *Aspergillus parasiticus* TISTR 3276 and *A. flavus* PSRDC-4, known for producing high levels of aflatoxins, have been previously identified (Boukaew et al. 2020b, c). For spore inoculum preparation, conidia of the two aflatoxin-producing fungi were harvested in 10 mL of sterile 0.1% (v/v) Tween 80 solution by gentle scraping of fungal mycelia and spores from potato dextrose agar plates (39 g/L; Difco Laboratory) at 30 ℃ of 7-day-old cultures.

Antagonism of *S. philanthi* RL-1-178 against the growth of the two aflatoxigenic fungal strains using a dual culture technique

S. philanthi RL-1-178 was evaluated for their antagonistic properties against *A. parasiticus* TISTR 3276 and *A. flavus* PSRDC-4 using a dual culture technique (Islam et al. 2009). A streak of spore suspension at 10^7 spores/mL of *S. philanthi* RL-1-178 was deposited on one side of a PDA medium in Petri dishes and incubation at 30 ℃ for seven days. A 5-mm-diameter mycelial plug, taken from a 2-day-old colony of each aflatoxigenic fungal strain, was placed in the center of each plate. A control was also performed by placing a mycelial plug of the aflatoxigenic fungal strain on a PDA plate without the addition of *S. philanthi* RL-1-178. The dual culture plates were then incubated at 30 ℃ for three days, and the radial growth of the aflatoxigenic fungal strain was compared to the control by measuring the mycelial growth. Three replicates were conducted for each *S. philanthi* RL-1-178–aflatoxigenic fungal strain combination. The colony size in each treatment was recorded and the percentage inhibition of hyphal growth was calculated.

Preparation and characterization of hydroxypropyl methylcellulose and polyvinyl pyrrolidone as composite film coating solution

Hydroxypropyl methylcellulose (HPMC) and polyvinyl pyrrolidone of K30 and K90 (PVP-K30 and PVP-K90) (Sigma Aldrich India) were selected as composite film. The film coating solution was prepared by dissolving each film in distilled water at 95 °C for 30 min while constantly stirring (120 rpm) to achieve a final concentration of 1.0, 2.0 and 3.0% (w/v) for HPMC and 1.0, 2.0, 3.0, 4.0, 5.0, and 10.0% (w/v) for PVP. The film coating solution of each concentration was determined for pH and viscosity. The pH and viscosity were measured using a pH meter (Mettler Toledo seveneasy, Switzerland) and a viscometer (Brook field digital viscometer, DV-III ULTRA, USA), respectively, at 25 ℃. The most suitable concentration and type of film were selected based on the pH, viscosity, and stability of the film solution, using criteria established by previous studies (Korkasetwit et al. 2008; Siri et al. 2010).

Preparation of film-coating maize grain and its morphology under SEM

The 100 g maize grain was coated with 1.0 mL of the film coating solution containing PVP-K90 or PVP-K90 (each at 4.0%, w/v) plus the DCF RL-1-178 (15%, v/v) solution. The maize grain and the film coating solution were mixed manually for 1.0 min, before drying in laminar airflow for 48 h (Vercelheze et al. 2019). The morphology of uncoated and coated maize grain was illustrated by scanning electron microscopy (SEM) employing a FEI Quanta 400 microscope (Hillsboro, Oregon, EUA). Prior to SEM visualization, maize grain from each treatment was dried in an air circulation oven at 35 °C for 24 h and kept in desiccators containing anhydrous calcium chloride for 1 week. The samples were then coated with gold, and images were taken using an acceleration voltage of 20 kV.

In vitro study on the inhibition efficacy of coated maize grain against the two aflatoxigenic fungal strains, and illustration of its interactions microstructure

Preparation of maize grain coating

Commercial maize grain was obtained from Pacific Seed Co., Ltd., Saraburi Province, Thailand. The film coating was carried out by using a rotary plate coating machine (P. Mitcharoen Machine Tool Co., Ltd., Thailand). The coatings were conducted as follows: initially, the DCF RL-1-178 was dissolved in distilled water to achieve the final concentrations of 0, 5.0, 10.0, and 15.0% (v/v). The PVP-K90 concentration at 4.0% (w/v) was prepared in distilled water at 95 °C for 30 min under constant stirring. The maize grain (1.0 kg) was coated with the film solution formulation in a ratio of 10:1 (w/v). Only PVP-K90 and commercial synthetic metalaxyl® were used as negative and positive control, respectively. Finally, the maize grain and coating solution were then mixed by stirring (at 725 rpm) in a rotary plate coating machine for 1.0 min, and the coated seeds were stored for 48 h until completely dry in laminar airflow before further use (Vercelheze et al. 2019).

In vitro study on the inhibition efficacy of coated maize grain against the two aflatoxigenic fungal strains

Efficacy of coated maize grain against *A. parasiticus* TISTR 3276 and *A. flavus* PSRDC-4 was investigated on a PDA Petri dish according to the method of Kaewkham et al. (2016) with some modification. Briefly, 20 µL of a spore suspension at 10^5 spores/mL of each aflatoxin-producing fungi was dropped on PDA in the center of a 9-cm-diameter Petri dish and then deposited each coated maize grain on the plate at four equidistance sites 2 cm apart from the center, using sterile forceps. The coated maize grains containing sterilized water and 35% WP metalaxyl® (Hitech Group Chemical Supply Co. Ltd) were used as a negative and positive control, respectively. The radial growth of aflatoxin-producing fungi was observed after incubation at 30 °C for 5 days. The colony size in each treatment was recorded and the percentage inhibition of hyphal growth was calculated using the equation: Percentage of inhibition = [(Control–Treatment)/Control] × 100. For each treatment, three replicates were conducted.

The morphology interaction of coated maize grain in treated and non-treated (control) (from the PDA Petri dish) against the two aflatoxigenic fungi was observed by microscope (Leica). To confirm the fungal interaction by SEM observation, the following procedures were adopted for the samples before microscopic observation according to the method of Tang et al. (2018) with some modification. Briefly, the coated maize grain in treated and non-treated (control) was first fixed with 1.5% glutaraldehyde and dehydrated with a graded series of ethanol (50–100%, 10% intervals) washes for 20 min followed by drying in a desiccator. Finally, the dried specimens were coated with gold and examined by SEM employing an FEI Quanta 400 microscope (Hillsboro, Oregon, EUA).

Efficacy of coated maize grains to prevent growth and aflatoxin B_1 production of the two aflatoxigenic fungal strains during 5 months of storage

The effectiveness of coated maize grains in preventing growth and aflatoxin B_1 (AFB_1) production by the two aflatoxigenic fungi in maize grains during 5 months of storage was evaluated using the method described by Afsharmanesh et al. (2018). Briefly, 10 mL of a spore suspension at 1×10^5 spores/mL of each aflatoxigenic fungal strain was aseptically placed in a sterile plastic bag containing 500 g coated maize grains and mixed gently for 2 min (modified from Krusong et al. 2015). The coated maize grains were then stored for 5 months under two conditions; at room temperature (28 ± 2 ℃ and 55 RH) and room temperature (28 ± 2 ℃) under 90% relative humidity (RH) (using $BaCl_2$ solution) (Rockland 1960). Then, the evidence of the growth and aflatoxin B_1 production of the two aflatoxigenic fungi after spreading on PDA plates and incubation at 30 ℃ was examined every month. Extraction of aflatoxin from the coated maize grain employed a modification of the method described by Sidhu et al. (2009). For aflatoxin B_1 production, samples after treatment were extracted with chloroform according to the Association of Official Analytical Chemists (Tosch et al. 1984) and AFB_1 concentration was determined as previously described by Boukaew et al. (2020c). AFB_1 concentration was determined by indirect competitive ELISA (Enzyme-linked Immunosorbent assay) using a ScreenEZ® Aflatoxin ELISA test kit (Siam Inter Quality Co., Ltd., Thailand).

To confirm the aflatoxin B_1 production at the end of 5 months of coated maize grain storage, aflatoxin concentration was determined using HPLC according to the method by AOAC (1984) at Central Laboratory (Thailand) Co., Ltd. The detection limit for determination of aflatoxin by HPLC was at 0–20 ppb.

Cytotoxicity assay

The cytotoxicity of the DCF RL-1-178 was evaluated *In vitro* using Vero cells (Elabscience, Wuhan, Hubei, China) according to the method described by Eawsakul et al. (2017). Vero cells were cultured in Dulbecco's Modified Eagle's (DMEM) medium (Merck KGaA, Darmstadt, Germany)

containing 10% (v/v) fetal bovine serum (FBS) and 1% antibiotic cocktail (penicillin G at 100 units/mL and streptomycin at 100 mg/mL). The culture was incubated at 37 ℃ in a humidified 5% (v/v) CO_2 95% air atmosphere. The medium was replaced every 2 days until the cells reached 90% confluence. The cells were then detached from the T-flask using a trypsin-ethylenediaminetetraacetic acid (EDTA) solution (0.25% trypsin, 1 mM EDTA) and further incubated at 37 ℃ in a humidified incubator (Binder, Tuttlingen, Germany) with 95% room air and 5% CO_2. After incubation, 100 µL of the suspensions were seeded in 96-well plates at a density of 10^5 cells/well, followed by the addition of 100 µL of DCF RL-1-178 at varying concentrations. Positive and negative controls were established by adding 1% (v/v) Triton X-100 and 0.01 M PBS solution pH 7.4 respectively in the same amount. After 24 h of incubation, the viability of the Vero cells treated with the solution was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The medium was removed, and the cells were incubated with fresh medium containing 0.5 mg/mL of MTT for 4 h. The formazan crystals formed were dissolved by adding 100 µL of DMSO. The absorbance was determined using a microplate reader (Metertech M965, Taipei, Taiwan) at 570 nm. The cell viability and cytotoxicity were calculated using the following equations, respectively: Cell viability (%) = (Absorbance value of treated cells − Absorbance value of blank)/(Absorbance value of untreated cells − Absorbance value of blank) × 100 (Chilczuk et al. 2020) and Cytotoxicity (%) = [(Absorbance value of untreated cells)—(Absorbance value of treated cells/Absorbance value of untreated cells)] × 100 (Hamilton 1978).

According to ISO 10993–5 standard, cell viability percentages above 80% are considered non-cytotoxic; between 80 and 60% is considered weak; between 60 and 40% is considered moderate, and below 40% is considered strong cytotoxicity (International Organization for Standardization ISO 10993–5, 2009).

Statistical analysis

All the experiments were done in replicates ($n = 3$), and the data were subjected to Analysis of Variance (ANOVA), (SPSS, version 21; IBM Corp, Armonk, NY). The study compared mean values and significant differences using Tukey's HSD test at a p-value of less than 0.05.

Results

Antagonism of *S. philanthi* RL-1-178 against the growth of the two aflatoxigenic fungal strains using the dual culture technique

The strong antifungal activity of *S. philanthi* RL-1-178 against the two aflatoxigenic fungal strains was confirmed on a PDA agar plate using the *In vitro* dual culture technique as illustrated in Fig. 1. After three days incubation at 30 ℃, *S. philanthi* RL-1-178 inhibited the mycelial growth of *A. flavus* PSRDC-4 effectively (95.0%) and a significantly difference ($p < 0.05$) from that against *A. parasiticus* TISTR 3276 (89.0%). The treatment without *S. philanthi* RL-1-178 was used as a control. The result also indicated that *A. flavus* PSRDC-4 was more susceptible to the antifungal agent of *S. philanthi* RL-1-178 than *A. parasiticus* TISTR 3276.

Physicochemical properties of composite film coating

The effect of hydroxypropyl methylcellulose (HPMC) and polyvinyl pyrrolidone (PVP-K30 and PVP-K90) content on the physicochemical properties (pH and viscosity) of composite polymer solutions is shown in Fig. 2. The increased

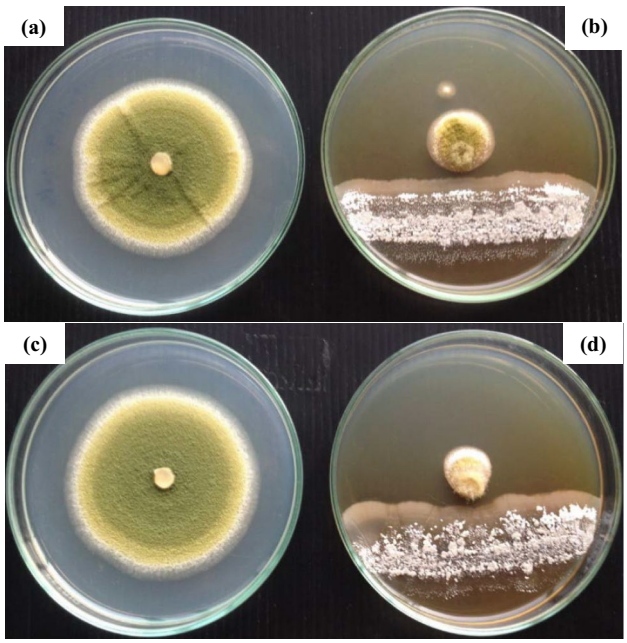

Fig. 1 Antifungal activity of *S. philanthi* RL-1-178 against the two aflatoxigenic fungal strains by using a dual culture technique on PDA agar plates and incubated for three days. **a** *A. parasiticus* TISTR 3276 (control), **b** *A. parasiticus* TISTR 3276 and *S. philanthi* RL-1-178, **c** *A. flavus* PSRDC-4 (control), and **d** *A. flavus* PSRDC-4 and *S. philanthi* RL-1-178

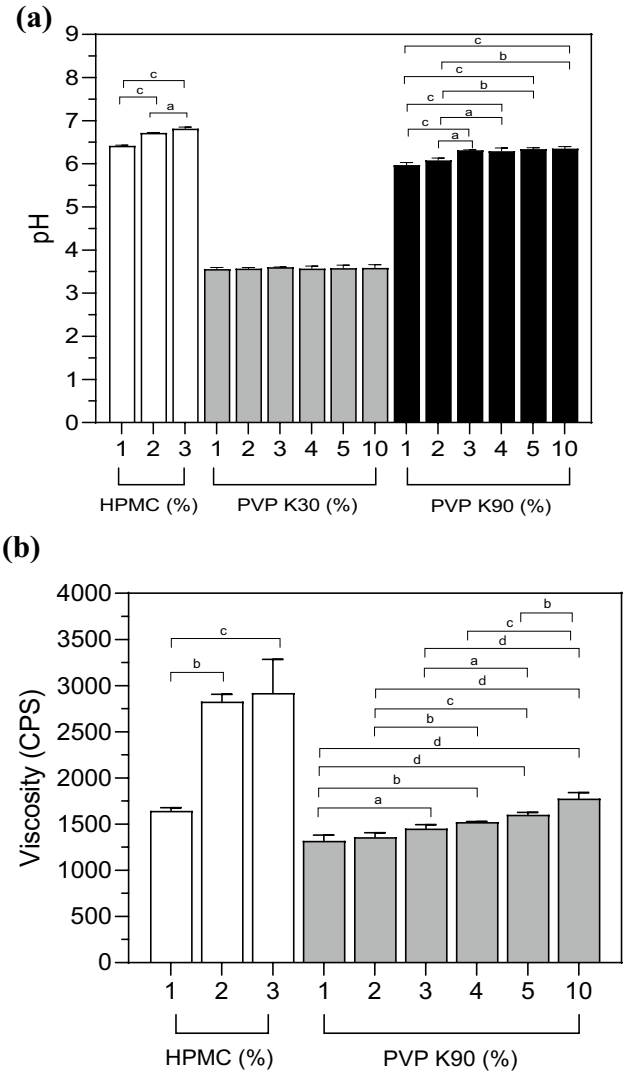

Fig. 2 pH **a** and viscosity **b** values of films solution determined before accelerated testing. The values are means of three replicates and their standard deviation

concentrations of HPMC and PVP-K90 content were responsible for the increased pH of composite polymer solutions, with HPMC and PVP-K90 values ranging from 6.4 to 6.7 and 6.0 to 6.4, respectively and significantly ($p < 0.05$) of PVP-K30 (pH from 3.54 to 3.60) (Fig. 2a). The optimal pH for the film-coating formulation was reported to be in the pH range of 5.5–7.0 (Korkasetwit et al. 2008). Therefore, only the samples from HPMC and PVP-K90 were selected and measured for viscosity. The viscosity of the composite film coatings increased significantly ($p < 0.05$) from 1633 to 2820 CPS for HPMC and 1307 to 1453 CPS for PVP-K90 when 2% HPMC and 3% PVP-K90 were added, respectively (Fig. 2b). High viscosity influenced corn coating formulation, while low viscosity affected consistency in corn coating formulation (Siri et al. 2010). Furthermore, it was suggested that the PVP K90 can be added to the seed coating formulation in even lower percentages, such as 1–4%, due to its higher viscosity. Therefore, PVP-K90 at a concentration of 4.0% (w/v) was selected for further studies.

Preparation of film-coating maize grain with and without the DCF RL-1-178 and their morphology under SEM

The coating homogeneity of PVP-K90 and PVP-K-90 mixed with the DCF RL-1-178 was illustrated by SEM, as shown in Fig. 3. Uncoated control maize grains (Fig. 3a-d) presented a slightly smooth surface and roughness. The PVK-90 applied to the coating resulted in a uniform layer over the maize grains surfaces, which appear fully coated and presenting smooth surfaces with no roughness or brittle points (Fig. 3e–h). The maize grains coated with the mixture between PVK-90 and the DCF RL-1-178 showed a smooth surface and no brittle points (Fig. 3i–l). SEM results indicated that the DCF RL-1-178 did not affect the maize grain coating formulation.

In vitro study on the inhibition efficacy of coated maize grain against *A. parasiticus* and *A. flavus*, and illustration of its interactions microstructure

The effect of coated maize grain with different concentrations of the DCF RL-1-178 [5.0, 10.0, and 15.0% (v/v)] on the inhibition of mycelial growth of the two aflatoxigenic fungal strains on PDA plates was investigated (Fig. 4). The mycelial growth by *A. parasiticus* TISTR 3276 and *A. flavus* PSRDC-4 was significantly ($p < 0.05$) inhibited in the range between 11.61 and 54.80% and 17.17–54.17% respectively (Fig. 4a). Coated maize grains with 15.0% (v/v) of the DCF RL-1-178 had the most profound effect on the growth of *A. parasiticus* TISTR 3276 and *A. flavus* PSRDC-4 with 54.80% and 54.17% inhibition, respectively. These were about two-fold more efficient than the fungicidal Metalaxyl® (28.06% and 26.94% inhibition, respectively). The mycelial growth interaction between coated maize grains of the two aflatoxigenicfungal strains was presented on PDA plates, as shown in Fig. 4b.

The microstructure of interactions between coated maize grain (with the DCF RL-1-178 (15% (v/v)) and control treatment) and the two aflatoxigenic fungal strains was observed using a microscope (Fig. 5). Many *A. parasiticus* TISTR 3276 (Fig. 5A; a, b) and *A. flavus* PSRDC-4 (Fig. 5B; a, b) mycelium and visible spores were distributed to cover maize grain in the control treatment but no growth and visible spores of *A. parasiticus* TISTR 3276 (Fig. 5A; c, d) and *A. flavus* PSRDC-4 (Fig. 5B; c, d) occurred in maize grain coated with the DCF RL-1-178. SEM also confirmed the microstructure of interactions between coated maize grain

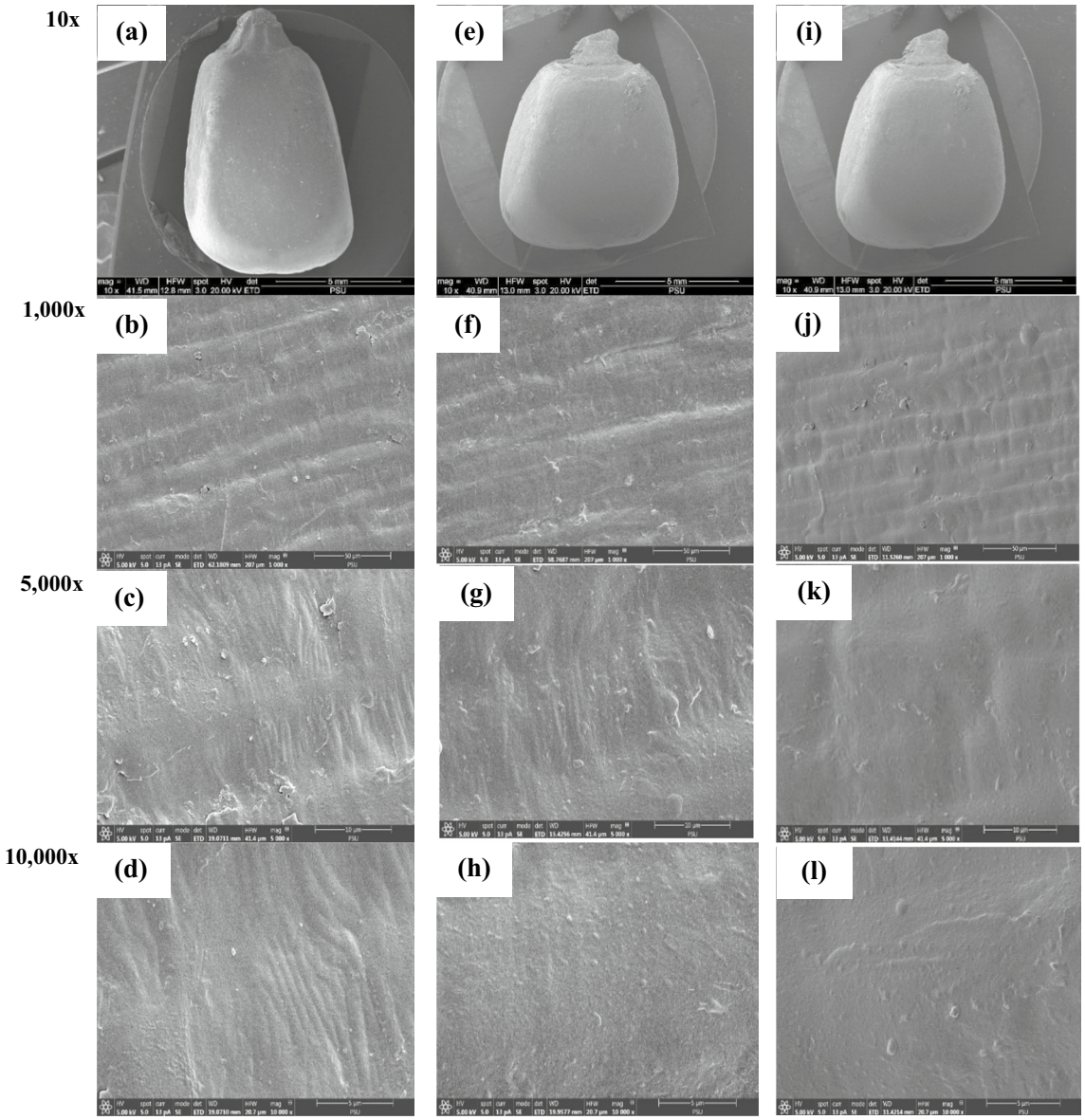

Fig. 3 SEM images of surface morphologies of maize grain; **a–d** uncoated and **e–h** coated with PVPK90 (4.0%, w/v) as well as **i–l** coated with PVK-90 plus DCF RL-1-178 (15%, v/v)

and the two fungal strains (Fig. 6). Similarly, resulting from a microscope, *A. parasiticus* TISTR 3276 and *A. flavus* PSRDC-4 affected the coated maize grain in the absence (control) or presence of the DCF RL-1-178. Therefore, coated maize grain with the DCF RL-1-178 (15%, v/v) was chosen for further studies.

Efficacy of coated maize grain to prevent growth and aflatoxin B_1 production of *A. parasiticus* and *A. flavus* during 5 months of storage

The efficacy of the coated maize grain to inhibit growth and AFB_1 production from the two aflatoxigenic fungal strains during 5 months of storage at room temperature (Fig. 7) was compared to those at room temperature under 90% RH (Fig. 8). Storage at room temperature, maize grain coated with distilled water (control) showed heavy growth and AFB_1 production of *A. parasiticus* TISTR 3276 (Fig. 7a, b) and *A. flavus* PSRDC-4 (Fig. 7c, d) in maize grain during storage, but neither growth nor AFB_1 production occurred in maize grain coated with the DCF RL-1-178 or Metalaxyl® for 5 months. For storage at room temperature under 90% RH, results of the control treatment on growth and AFB_1 production of *A. parasiticus* TISTR 3276 (Fig. 8a, b) and *A. flavus* PSRDC-4 (Fig. 8c, d) were similar to those in storage at room temperature.

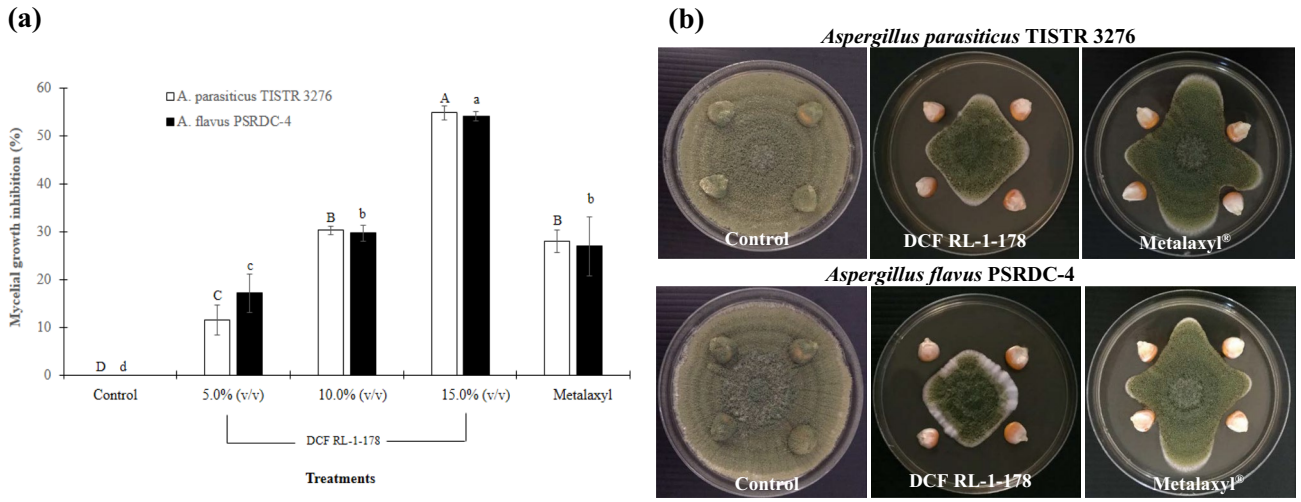

Fig. 4 The mycelial growth inhibition **a** of maize grain coated with various concentration of the DCF RL-1-178 and the commercial fungicide Metalaxyl® against *A. parasiticus* TISTR 3276 and *A. flavus* PSRDC-4, and **b** on PDA agar plates after incubation at 30℃ for seven days

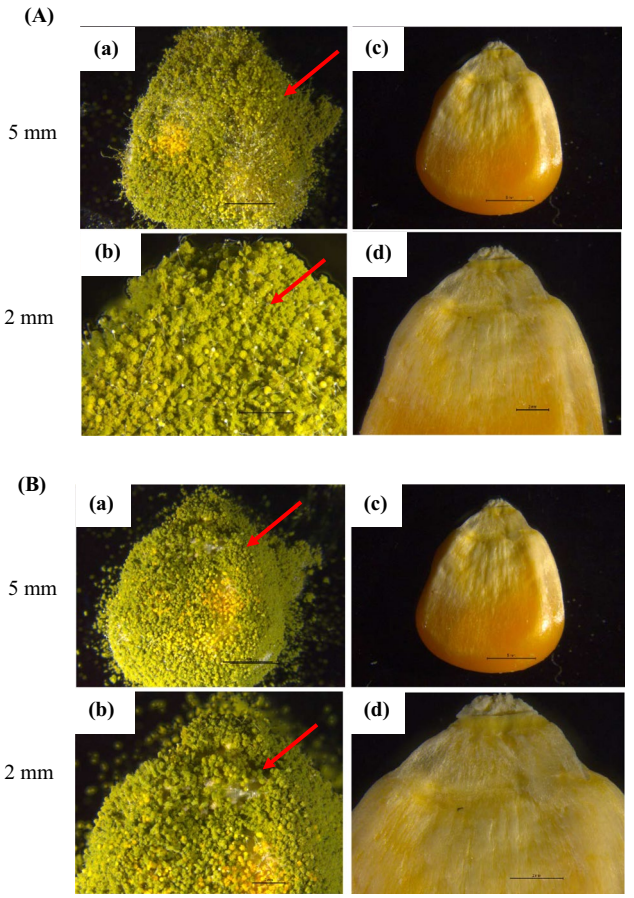

Fig. 5 Microscope images of **A** *A. parasiticus* TISTR 3276 and **B** *A. flavus* PSRDC-4 growing on coated maize grain in the absence (control) **a, b** or presence **c, d** of the DCF RL-1-178 (15.0%, v/v). Red arrows indicate the two aflatoxin-producing fungi growing on coated maize grain without the DCF RL-1-178 (control)

The significant ($p < 0.05$) control growth and AFB_1 production of maize grain samples between the DCF RL-1-178 and Metalaxyl®. Maize grains coated with the DCF RL-1-178 results demonstrated that the growth (Fig. 8a, c) and AFB_1 production (Fig. 8b, d) of maize grain samples controlled stable growth and AFB_1 production for only 3 months while Metalaxyl® could control for 4 months. After storage for 3 months, the increase in growth of *A. parasiticus* TISTR 3276 (from 0 to 5.97 log CFU/g) and *A. flavus* PSRDC-4 (from 0 to 4.77 log CFU/g) on maize grain samples coated by the DCF RL-1-178 was significantly ($p < 0.05$) higher than the increase of *A. parasiticus* TISTR 3276 (from 0 to 3.85 log CFU/g) and *A. flavus* PSRDC-4 (from 0 to 3.42 log CFU/g) on maize grain coated by the Metalaxyl®.

To confirm the ability of the coated maize grain to inhibit aflatoxin B_1 production from *A. parasiticus* TISTR 3276 and *A. flavus* PSRDC-4, samples from the two conditions were taken after 5 months of storage and analyzed for AFB_1 by HPLC (Table 1). Inoculated coated maize grains (control) and storage at room temperature and room temperature under 90% RH showed that *A. flavus* PSRDC-4 (208.27 and 392.40 ug/kg, respectively) produced aflatoxin B_1 higher than *A. parasiticus* TISTR 3276 (146.65 and 213.80 ug/kg, respectively). AFB_1 production of the two aflatoxin-producing fungi was not detected in the inoculated coated maize grain with the DCF RL-1-178 or Metalaxyl® after storage at room temperature. On the contrary, the inoculated maize grain and storage at room temperature under 90% RH showed higher AFB_1 production than that produced in the storage at room temperature.

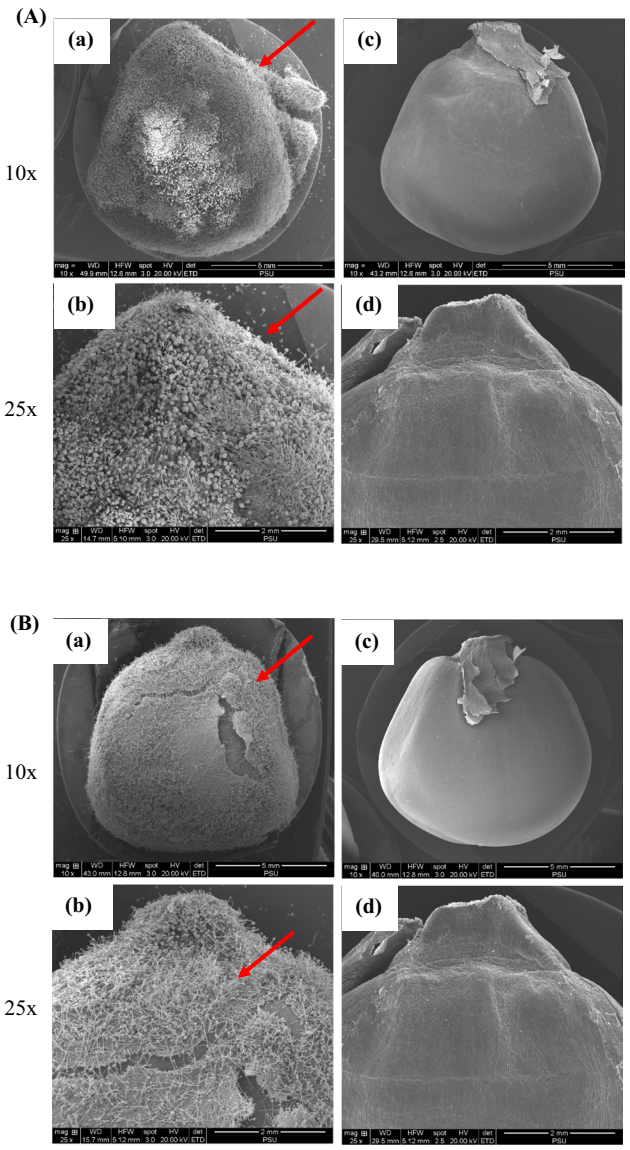

Fig. 6 SEM images of **A** *A. parasiticus* TISTR 3276 and **B** *A. flavus* PSRDC-4 growing on coated maize grain in the absence (control) **a, b** or presence **c, d** of the DCF RL-1-178 (15.0%, v/v). Red arrows indicate the two aflatoxin-producing fungi growing on coated maize grain without the DCF RL-1-178 (control)

In vitro cell cytotoxicity

The potential toxicity of DCF RL-1-178 on Vero cells was evaluated using the MTT assay to determine its cytotoxicity. According to ISO 10993–5, a compound is considered non-cytotoxic if it results in cell viability exceeding 80%, while a cell viability below 40% is considered strongly cytotoxic. In the present study, the non-cytotoxic dose of the DCF RL-1-178 was determined to be at or below a concentration of 10 µg/mL, while the strongly cytotoxic concentration was found to be at or above a concentration of 100 µg/mL (Fig. 9). Thus, the results of this study confirm that DCF RL-1-178 at a concentration of 15% (v/v) or 1000 µg/mL had a strong cytotoxic effect on Vero cells.

Discussion

Mycotoxins producing fungi can invade the food and feed supply during production, processing, transportation, or storage. Thus, coating animal feed raw-material including maize with curing materials, especially those containing antifungal compounds, could help in the healing and protection of grains through the formation of additional barriers toward fungal pathogens. The present study focused on the antifungal effects of the DCF RL-1-178 derived compounds of *Streptomyces philanthi* RL-1-178 to be used as maize grain coating to control *A. parasiticus* and *A. flavus*, using both *In vitro* and maize grain storage. Coating materials used on grains must maintain their structural integrity and prevent cracks and breaks on the grain surface after drying, as well as during transportation and handling (Sharma et al. 2015). The SEM images showed that the maize grain coated with PVP-K90 and PVP-K90 mixed with DCF RL-1-178 had a uniform layer over the grain surfaces, appearing fully coated with smooth surfaces and no roughness or brittle points. The results of the morphology surface showed that the presence of DCF RL-1-178 did not affect the maize grain coating formulation. This suggests that the DCF RL-1-178 coating not only acts as an antifungal coating, but also as a carrier system for agrochemicals, which reduces losses due to environmental factors. This property could lead to a reduction in the amount of fungicides needed, resulting in a reduction of environmental pollution. Aflatoxin contamination can occur in various crops and food products, including maize, rice, spices, dried fruits, nuts, and figs, which are commonly affected (Martinez-Miranda et al. 2019). The use of antifungal metabolites in the form of DCF for maize coating to inhibit the growth of aflatoxigenic fungal strains has not been reported previously. However, the potential benefits of using this bioactive agent as a coating treatment for maize grains to prolong storage duration by preventing fungal growth and mycotoxin production is worth investigating. The present study is the first to demonstrate that incorporating DCF RL-1-178 into a maize coating formula is effective in controlling postharvest disease caused by *A. parasiticus* TISTR 3276 and *A. flavus* PSRDC-4 on maize grain. This study demonstrated that incorporating the antifungal compound DCF RL-1-178 into a maize coating formula can effectively control postharvest fungal growth and mycotoxin production caused by *A. parasiticus* TISTR 3276 and *A. flavus* PSRDC-4 on maize grain. Results showed that varying levels of DCF RL-1-178 concentrations in the coating formula improved its effectiveness against fungal growth, as observed by reduced *In vitro* radial growth. The *In vitro*

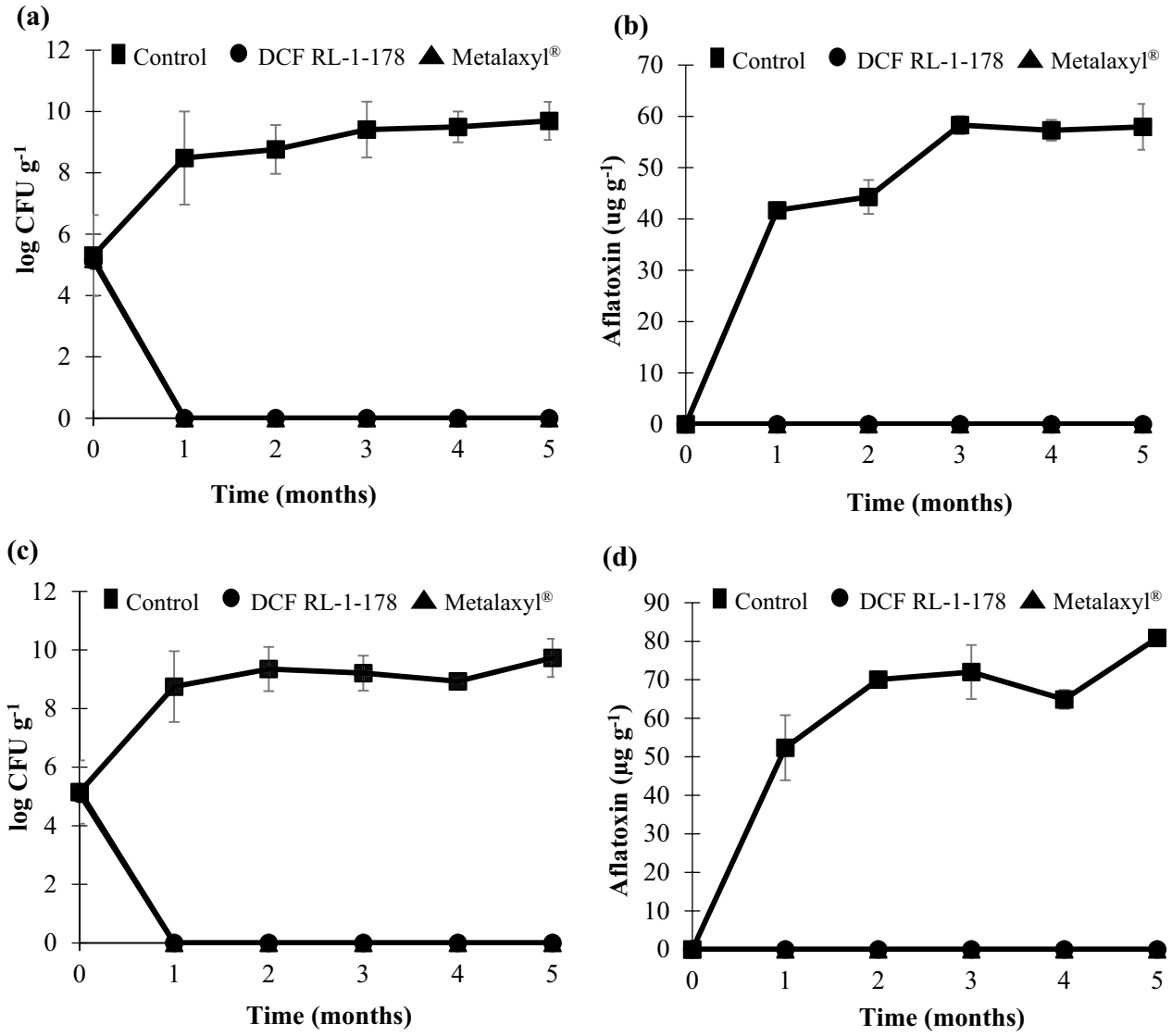

Fig. 7 Efficacy of coated maize grain to prevent on growth and AFB_1 production of **a, b** *A. parasiticus* TISTR 3276 and **c, d** *A. flavus* PSRDC-4 in maize grain during storage at room temperature for five months. The values are means of three replicates and their standard deviation

results indicated that the DCF RL-1-178 coating at the highest concentration (15%, v/v) was about two folds more effective than the synthetic fungicide Metalaxyl® in controlling the disease, although the difference was significant. Two-fold increase of the DCF RL-1-178 concentrations from 5.0 to 10.0% (v/v) resulted in a two-fold increase in growth inhibition efficacy. Thus, it could be concluded that the antifungal metabolites of *S. philanthi* RL-1-178 could be applied in the form of DCF for maize coating formula and replaced the use of synthetic fungicides. This finding suggested a simpler process to produce and apply antifungal compounds in the crude form using freeze-dried culture filtrate (DCF) incorporation into a maize coating formula that was able to prevent *Aspergillus* species in maize grain. This result was in line with Morcia et al. (2012), who found that the fungistatic or fungicide effect was highly dose-dependent. Therefore, it could be concluded that the antifungal activity of the integral DFC RL-1-178 in coated maize may be attributable to the active components of the DCF RL-1-178. They were anticipated to be antibiotics as *Streptomyces* are known to produce some antifungal drugs. Amphotericin B (AMB), produced by *S. nodosus*, is the broad spectrum antifungal drug active against most fungi, molds, and yeasts (Sharma et al. 2012). To the best of our knowledge, this is the first published report of coated maize grain incorporating fungal bioactive agents (DCF RL-1-178) exhibiting fungicidal activity in dual culture experiments on the two aflatoxigenic pathogens. The concentrations found to be effective in *In vitro* tests were taken as the baseline to derive the treatments for the maize storage.

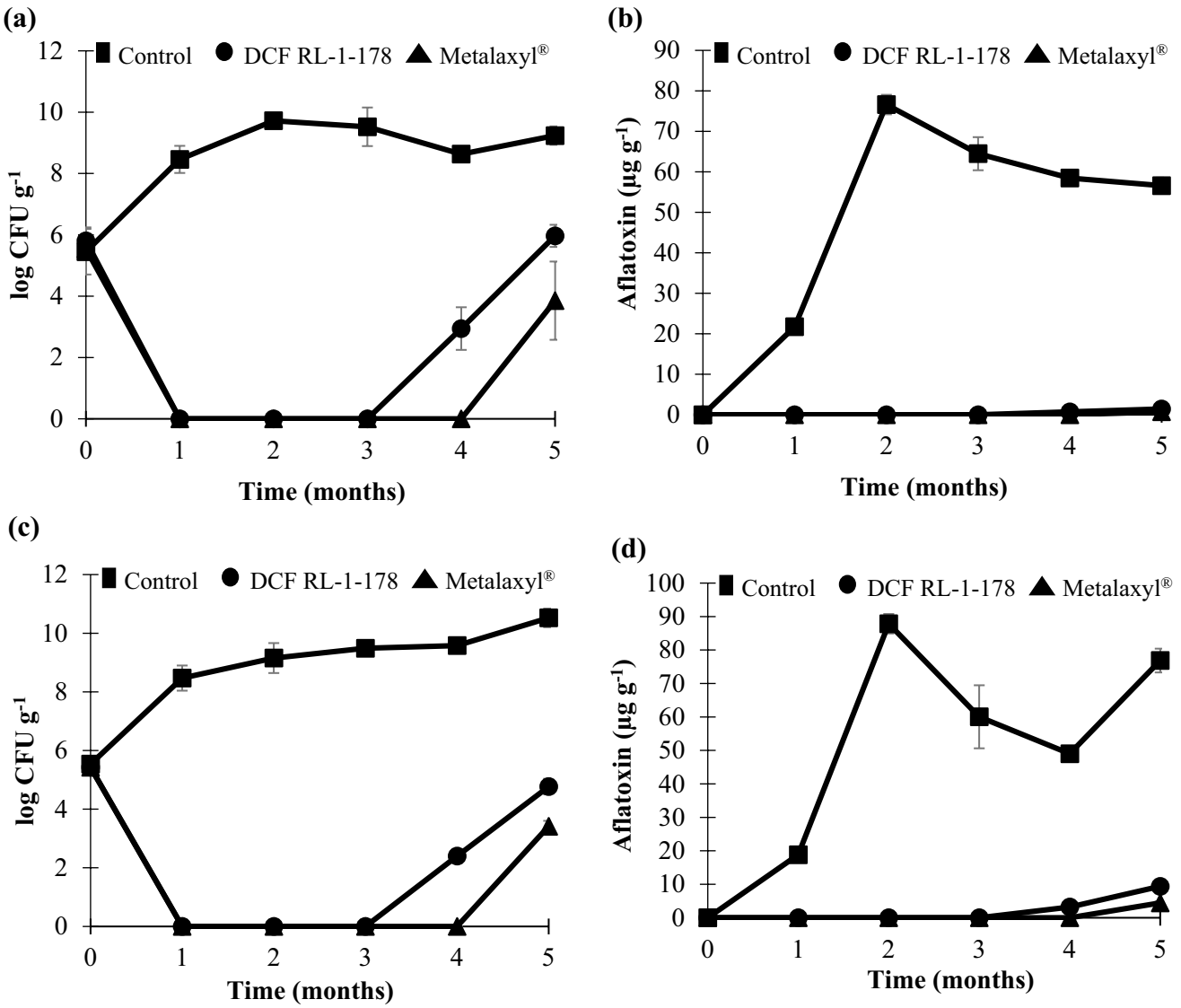

Fig. 8 Efficacy of coated maize grain to prevent on growth and AFB_1 production of **a, b** *A. parasiticus* TISTR 3276 and **c, d** *A. flavus* PSRDC-4 in maize grain during storage at room temperature with 90% relative humidity for five months. The values are means of three replicates and their standard deviation

Table 1 Effect of coated seeds on aflatoxin B_1 production of *A. parasiticus* TISTR 3276 and *A. flavus* PSRDC-4 detected by HPLC after stored at room temperature and room under 90% relative humidity for five months

Seed coating material	Aflatoxin B_1 production (µg kg^{-1}) by *A. parasiticus* TISTR 3276		Aflatoxin B_1 production (µg kg^{-1}) *A. flavus* PSRDC-4	
	Stored at room temperature	Stored at room temperature under 90% relative humidity	Stored at room temperature	Stored at room temperature under 90% relative humidity
Control	146.65	213.80	208.27	392.40
DFC RL-1-178	Not detected	47.43	Not detected	92.82
Metalaxyl®	Not detected	3.76	Not detected	14.28

The use of chemical compounds in grain protection is becoming less desirable, and research into the use of microorganism-derived compounds, such as those produced by *Streptomyces*, as an alternative method for controlling fungi in maize grain before and after harvest, as well as during storage, is increasing in popularity (Chulze 2010; Luo et al.

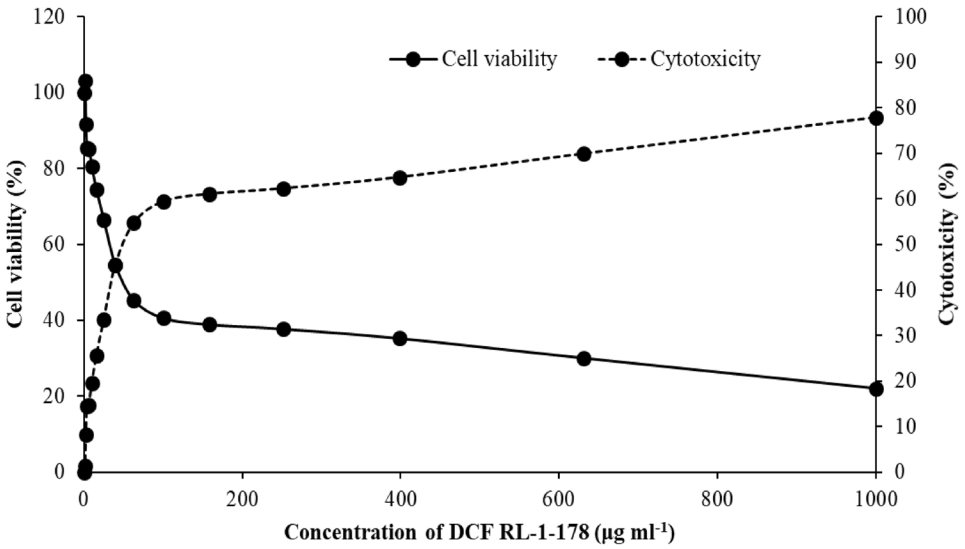

Fig. 9 *In vitro* cytotoxic effect of the DCF RL-1-178 against Vero cells. The cytotoxic activity of the DCF RL-1-178 on Vero cells was evaluated after incubation at 37 ℃ for 24 h

2018). Several studies have reported that the complete eradication of pathogens with biofungicides under seed storage is challenging, although many substances can completely inhibit fungal growth *In vitro* (Cheong et al. 2018; Aliabbasi et al. 2021). The incorporation of DCF RL-1-178 into a PVP-K90 coating that could potentially treat and protect maize grain from the aflatoxigenic strains infection was also investigated in maize storage. Compared to DCF RL-1-178 or Metalaxyl® coating, DCF RL-1-178 (15%, v/v) coating was the most effective treatment and reduced both pathogens on maize gain comparable to the formulation of Metalaxyl®. Based on the results of the maize grain storage, it is very interesting that maize grain coating incorporating a bioactive agent in the DCF RL-1-178 (15%, v/v) could protect mold and AFB_1 production of *A. parasiticus* TISTR 3276 and *A. flavus* PSRDC-4 during 5 months of maize grain storage at room temperature equal to synthetic fungicide Metalaxyl®. Therefore, it could be concluded that the mechanism of coated maize interaction between the aflatoxigenic fungal strains may be direct antibiosis DCF RL-1-178. The results of the storage experiment at room temperature with 90% relative humidity indicated that the number of *A. flavus* PSRDC-4 in the control group increased from 2 to 4 months, while the aflatoxin content decreased. This discrepancy may be attributed to other factors such as nutrient availability and competition from other microorganisms that could be influencing the production of aflatoxin by *A. flavus* PSRDC-4. Alternatively, the decrease in aflatoxin content could be due to degradation or absorption of the toxin by the maize grain over time. Further research is required to better comprehend the underlying mechanisms behind the observed trends in *A. flavus* PSRDC-4 growth and aflatoxin production. This may involve investigating the impact of various environmental conditions, such as temperature and humidity, on the growth and toxin production of *A. flavus* PSRDC-4, as well as exploring potential approaches for controlling or mitigating aflatoxin contamination in stored maize grain.

Our study focused on the use of microorganisms and their derived compounds through seed coating to prevent the growth of aflatoxigenic fungi and aflatoxin contamination in food and feedstuffs, while previous studies have primarily focused on promoting crop growth, yield, and protection against pathogens (Mancini and Romanazzi 2014; Ma et al. 2019; Cortés-Rojas et al. 2021; Mongiano et al. 2021). For that reason, the DCF RL1-178 maize coating was developed to incorporate biopolymer onto the maize grain surface to achieve protection against mold diseases. Therefore, maize coating formula was found to show significant anti-fungal growth, hence, it could be explored as with biofungicides for preventing microbial deterioration and mycotoxins accumulation in food and feedstuffs during pre- and post-harvest and storage.

During storage, the moisture content is the factor affecting grain quality (Lawrence and Maier 2011). Grains will lose their moisture to the surrounding airwhen the storage temperature increases, leading to an increase in relative humidity (RH) (Shah et al. 2002). In addition, the fluctuation of RH during storage could affect fungal growth and mycotoxin production, and cause considerable nutrient losses in grain (Rehman et al. 2002). Storage at 90% RH revealed that maize grain coated with the DCF RL-1-178 (3 months) could protect the growth and AFB_1 production of the two aflatoxin-producing fungi for three months which was lower than that coated with the Metalaxyl® (4 months). The results agreed with a few previous studies that the rapid growth of *A. flavus* and aflatoxin production were related to high RH levels (at 90–98% RH) (Pratiwi et al. 2015; Jaibangyang et al. 2021). The coating with the DCF RL-1-178 of composite films can protect against the two aflatoxin-producing fungi infections of maize grain for 5 months in comparison

with the uncoated DCF RL-1-178. Therefore, the composite film based on the DCF RL-1-178 can be utilized to extend the shelf life of maize grain which could beneficial in protecting seed pathogens and maintaining a good quality of maize grain during storage. The results indicated that the DCF RL-1-178 is a promising biofungicide that could be an alternative to synthetic fungicides. It is adapted to local environmental conditions, is easily and cheaply produced, and can be stored in a low-cost formulation. In addition, the cytotoxicity of the DCF RL-1-178 at a concentration of 15% (v/v) or 1000 µg/mL towards Vero cells was investigated. For this reason, the maize coating formula was determined based on the toxicity of the DCF RL-1-178 when used in maize grain coating, before it could be used as animal feed. The cytotoxicity assay revealed that DCF RL-1-178 (15% (v/v) or 1000 µg/mL) had a significant cytotoxic effect on Vero cells. These results indicate that DCF RL-1-178 could be an effective biofungicide for controlling maize seed storage, however, it should not be used for maize grain storage as animal feed.

Many studies have demonstrated *Streptomyces* as a rich source of bioactive compounds with antifungal activity against *Aspergillus* sp. (Caceres et al. 2018; Kemung et al. 2018; Shakeel et al. 2018; Campos-Avelar et al. 2021). Many mechanisms have been proposed to explain the antifungal activity of bioactive compounds of *Streptomyces* sp.; the first suggested that these compounds may disrupt the structure and composition of fungal cell walls, leading to lysis of hyphae or spores (Mander et al. 2016; Vurukonda et al. 2018) and may affect intracellular activities of mitochondria, cytoplasmic membrane, and nucleus (Gálvez-Iriqui et al. 2019; Kumar et al. 2021; Siupka et al. 2021). Another explanation indicated that the bioactive compounds could down-regulating the expression of aflatoxin-synthesis related genes (Yoshinari et al. 2007; Caceres et al. 2018). Metalaxyl® is a fungicide from the acylalanine group that is used to control plant diseases. It works by penetrating fungal cells and selectively disrupting DNA synthesis, which stops the growth of mycelium and prevents the formation of spores and haustoria (Fisher and Hayes 1984).

Contamination of foods and feedstuffs with AFB_1 is a public health concern because of the ability of AFB_1 to cause human and animal diseases (Mutungi et al. 2008; Dövényi-Nagy et al. 2020). The Codex Alimentarius Commission, established jointly by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO), is the main intergovernmental agency responsible for protecting consumer health and facilitating trade through the development of international standards for food and feed. Therefore, measurements of the AFB_1 concentration were confirmed by HPLC at the end of 5 months of maize grain storage. In control, uncoated maize grain inoculated with aflatoxigenic strains, *A. parasiticus* TISTR 3276 and *A. flavus* PSRDC-4, total aflatoxin was significantly produced in storage conditions. AFB_1 contamination on maize grain did not occur in the condition that the maize grain was coated with either the DCF RL-1-178 and stored at room temperature but the AFB_1 content increased to 47.43–92.82 µg/kg when stored at room temperature under 90% relative humidity. The AFB_1 content of the two aflatoxigenic fungi was higher than the maximum permitted levels for food and feed according to public health guidelines (> 20 µg/kg) (Dövényi-Nagy et al. 2020). The United States Food and Drug Administration (FDA) states that the acceptable levels of aflatoxins in food products can vary depending on the commodity and country. For example, the EU allows a maximum of 0.1–12 µg/kg AFB_1, the USA allows 20 ppb total AF, China allows 5–20 µg/kg AFB_1, and Thailand allows 20 ppb total aflatoxins in food products (Battilani et al. 2016; Panrapee et al. 2016; US Grain Council 2019; Tumukunde et al. 2020). This study found that using bioactive agents in the DCF RL-1-178 coating for maize grain resulted in a significant decrease in pathogenic grain spoilage fungi and could serve as a biological control for preventing food spoilage during storage. We successfully applied these bioactive agents in the DCF RL-1-178 coating to inhibit the growth of aflatoxin-producing fungi during maize grain storage.

In conclusion, film-coating of cereals is an emerging technology with increasing acceptance of its reliability in protecting cereals pre- and post-harvest fungal spoilage infection. This series of studies demonstrated that coating maize grains with a PVP-K90 containing bioactive agents in the DCF RL-1-178 were effective to prevent *A. parasiticus* and *A. flavus* for up to 5 months during storage and equal to commercial fungicide (Metalaxyl®). It was considered to be a promising new alternative method to be used in cereals product coating. These findings indicate that maize grain coating can be an effective method for delivering biocontrol agents to reduce aflatoxin contamination in maize grains. However, it should not be used for animal feed grain coating.

Author contribution SB: conceptualization, data curation, supervision, formal analysis, funding acquisition, investigation, methodology, writing—original draft, writing—review & editing. PM: conceptualization, investigation, formal analysis. WP: writing—review & editing. SS: writing—review & editing. SS: investigation, Writing—review & editing. JC: investigation, writing—review & editing. PP: funding acquisition, writing—original draft, writing, writing—review & editing.

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Data availability Not applicable.

Declarations

Competing interests The authors declare no competing interests.

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