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Curcumin-based photosensitization inactivates *Aspergillus flavus* and reduces aflatoxin B1 in maize kernels

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ABSTRACT

Different methods have been applied in controlling contamination of foods and feeds by the carcinogenic fungal toxin, aflatoxin, but nevertheless the problem remains pervasive in developing countries. Curcumin is a natural polyphenolic compound from the spice turmeric (*Curcuma longa* L.) that has been identified as an efficient photosensitiser for inactivation of *Aspergillus flavus* conidia. Curcumin mediated photoinactivation of *A. flavus* has revealed the potential of this technology to be an effective method for reducing population density of the aflatoxin-producing fungus in foods. This study demonstrates the influence of pH and temperature on efficiency of photoinactivation of the fungus and how treating spore-contaminated maize kernels affects aflatoxin production. The results show the efficiency of curcumin mediated photoinactivation of fungal conidia and hyphae were not affected by temperatures between 15 and 35 °C or pH range of 1.5–9.0. The production of aflatoxin B_1 was significantly lower ($p < 0.05$), with an average of 82.4 μg/kg as compared to up to 305.9 μg/kg observed in untreated maize kept under similar conditions. The results of this study indicate that curcumin mediated photosensitization can potentially be applied under simple environmental conditions to achieve significant reduction of post-harvest contamination of aflatoxin B_1 in maize.

1. Introduction

Mycotoxins are toxic secondary metabolites of some fungi, which can occur widely and variably as contaminants in foods and feeds (Smith et al., 2016) with significant health and economic impacts worldwide (Rodrigues and Naehrer, 2012). Humans and animals are exposed to mycotoxins mainly through ingestion of contaminated foods (Zain, 2011) leading to various health problems including instant death in acute cases, or cancers, immunosuppression and retarded growth in children, with prolonged exposure (IARC, 1993; Probst et al., 2007; Varga et al., 2009; Wannop, 1961; Williams et al., 2004). Occurrence of mycotoxins in food and feed is also associated with significant economic losses as a result of increased cost of production, lowered animal production, decreased market values, and irregularity of production (Cardwell et al., 2001).

Reducing mycotoxin contamination in food and feed is done by focusing on either preventing fungal colonisation and mycotoxin production, or removing the toxins from products by detoxification (Temba et al., 2016a,b). Fungal infection and accumulation of mycotoxins incrops occur both in the field and in storage (Ismaiel and Papenbrock, 2015). Applying fungicides and pesticides is a common preharvest remedy, while inactivation of fungi by thermal, chemical or photo-irradiation procedures are applied for postharvest control (Begum et al., 2009; Luksiene et al., 2005; Nemţanu et al., 2014). With no single method successful in combating the mycotoxin problem entirely, it is advocated to integrate various intervention measures depending on circumstances. Whereas promising achievements are reported on the use of photosensitization against pathogenic microbes (Luksienė and Zukauskas, 2009) application of the process to food microbes is an emerging science (Buchovec et al., 2016; Luksiene, 2005; Luksiene et al., 2005).

Photosensitization kills microbial cells through cytotoxic reactions induced by energy from photosensitiser molecules excited by light (Wainwright, 1998). Effective excitation of photosensitiser molecules is

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achieved when light of appropriate wavelength is used (Yoon et al., 2013). Application of photosensitization mediated by natural product dyes is more suitable than using synthetic products that might compromise consumer preferences (Hempel and Hamm, 2016). Some plants extracts are potential photosensitisers (Dodge and Knox, 1986), and are viewed as preferable photosensitisers in food applications (Aponiene et al., 2015).

Previous studies have reported the potential of using turmeric extract (curcumin) as a plant-originated photosensitiser in microbial decontamination (Dovigo et al., 2013; Hegge et al., 2012; Verwanger et al., 2011). We recently reported the efficacy of curcumin extract in mediating photoinactivation of conidia of the aflatoxin-producing fungus *Aspergillus flavus*, both in culture and when inoculated on maize kernels (Temba et al., 2016a,b). Ideally, photosensitisers should be safe for human consumption, cost-effective, biochemically stable, and adequately activated using light of appropriate wavelength (Allison et al., 2004).

Curcumin ((*E,E*)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is a polyphenolic compound obtained from the tubers of *Curcuma longa* (turmeric) as a yellow extract (Verghese, 1993). Curcumin-mediated photosensitization has been studied and characterized in a number of microbes including fungi such as *A. flavus* (Temba et al., 2016a,b), and *Candida albicans (*Dovigo et al., 2011a*)*. The extract has also shown photoinactivation efficiency against bacteria such as *Staphylococcus epidermidis* (Hegge et al., 2012), *Streptococcus mutans* (Paschoal et al., 2013), *Escherichia coli, Enterococcus faecalis,* and *Streptococcus intermedius* (Haukvik et al., 2009). In our previous study (Temba et al., 2016a,b) it was demonstrated that curcumin mediated photosensitization is an efficient technique for reducing *A. flavus* conidia populations in maize kernels. Reduction in the number of viable spores was observed when the spores were treated with a combination of light and curcumin, but not with either light or curcumin alone. It is however understood, that in addition to conidia, hyphae are an important vegetative fungal stage for propagation and aflatoxin production. Successful decontamination of both conidia and hyphae and limiting aflatoxin B_1 accumulation provide useful information on the utility of the technique as a postharvest method of aflatoxin control.

In reference to the previous work and to the best of our knowledge, the effectiveness of curcumin mediated photosensitization against hyphae of *A. flavus* has not previously been reported. In the real world situation, varying conditions of temperature and pH are expected when the technique is applied under different home and industrial conditions, and the impact of these parameters on curcumin-mediated photosensitization have not previously been documented.

This study was conducted to address the effectiveness of the photosensitization technique on deactivation of fungal hyphae and conidia under varying temperature and pH conditions. The study also investigates the effects of photosensitization on aflatoxin B_1 accumulation on experimentally infected maize kernels to predict the usefulness of the technique in controlling this toxin.

2. Materials and methods

2.1. Study design

The study was carried out in two sets of experiment. In the first experiment, an *in vitro* study to test photodegradation of *A. flavus* conidia and hyphae under varied temperature and pH was done. The second experiment was an *in vivo* experiment to compare aflatoxin B_1 production in photosensitization treated and untreated maize kernels.

2.2. Light source and photosensitiser

The source of light for photosensitization was an exon arc lamp machine (Polilight 500®, Rofin Australia Pty Ltd) with adjustable wavelength selection, and illumination was done in a light impenetrable cabinet. Light dose was selected by adjusting illumination time, and was calculated dependent on specific power indicated for each wavelength. The light dose used for this experiment was 60 J cm^{-2} at wavelength of 420 nm which in the previous study (Temba et al., 2016a,b) was observed to be effective for adequate fungal conidia photoinactivation.

Stock solution (100 μM) of natural curcumin (Sigma Aldrich, St. Louis, MO, USA) was prepared by first dissolving 5.3 mg curcumin in 6 ml absolute ethanol and then diluted with phosphate buffer saline solution (PBS; pH 7.4) to required volume.

2.3. Fungal materials

Aspergillus flavus reference strain (ATCC 28862) was used as source of conidia and hyphae. Conidia were harvested from five day old fungal colonies, grown using the stock conidia on malt extract agar (MEA) at 26 °C. Conidia harvest was done by carefully flooding the culture with 10 ml 0.1% Tween 80 solution and gently swirling for 1 min to bring the conidia into suspension. The suspension was decanted and centrifuged at 1000 relative centrifugal force (rcf) for 1 min to concentrate the conidia and reduce hyphae contamination. The supernatant was discarded and the conidia pellet was washed twice with 0.1% Tween 80. The pellet was resuspended in sterile distilled water. Conidia concentration was determined by Colony Forming Units (CFU) count conducted using a Colony Counter (Stuart Scientific, United Kingdom) after culture growth in MEA for 48 h. The harvested conidia were diluted into stock suspension with approximately 10^{13} conidia per millilitre which was stored at 4 °C and used within 4 weeks.

Fungal hyphae were obtained by inoculating 9.9 ml nutrient broth with 100 μL of 5×10^{12} fungal conidia suspension (0.015% agar, 0.03% meat extract in 0.05% peptone water) in Eppendorf tubes at 37 °C. After ca. 48 h, woolly mass of hyphae were harvested from the tubes using sterile wire loop. The hyphae were re-suspended in 1.5 mL sterile distilled water in new Eppendorf tubes and vigorously shaken for 1 min to obtain a uniform hyphal suspension. The hyphae were washed twice with sterile water and concentration determined by Colony Forming Units (CFU) count, as before. The stock suspension was stored at 4 °C and used within four weeks.

2.4. Effect of pH on photosensitization efficiency

The efficiency of *in vitro* photoinactivation of *A. flavus* conidia and hyphae at different pH levels was tested. Two millilitres of conidia and hyphae suspension at initial concentration of ca.8×10^4 CFU ml^{-1} were mixed with 2 ml curcumin solution (100 μM) to make a final volume of 4 ml and 50 μM curcumin in a 35 mm × 10 mm petri dish. The pH of the solutions after adding conidia/hyphae to the photosensitiser were measured using pH-meter (PHM210®, France).

A set of 48 petri dishes for conidia/photosensitiser suspension, and the same number for hyphae were prepared. Six groups of eight petri dishes each were formed from the set with each group adjusted to a specific pH level before illumination. The pH levels used were 2.5, 3.0, 5.5, 7.0, 9.0 and 12.5. Regulation of the solution pH to acidic was done by adding drops of 6 M hydrochloric acid (LabChem, Thermofisher Scientific Vic. Australia) and to alkaline by adding drops of 10 M sodium hydroxide (LabChem Thermofisher Scientific, NSW Australia). For each group, four petri dishes were illuminated with light of 420 nm wavelength at dose of 60 J cm^{-2} (PS + /L +) and the remaining four control petri dishes were directly inoculated to media without illumination (PS + /L-). Illumination was done by placing a petri dish (lidded) approximately 2 cm below the light bulb with constant stirring using a magnetic stirrer. The suspension temperature was measured with a digital thermometer (Comark C26®, Comark USA) before and immediately after each light treatment.

Duplicate aliquots of 100 mL from the treatment petri dishes were inoculated on MEA and incubated at 26 °C for 48 h. The controls (PS

+/L-set) were diluted at a ratio of 1:100 before plating on the growth media to enable counting of the colonies as a high CFU concentration was expected.

2.5. Effect of temperature on photosensitization efficiency

Two millilitres of conidia and hyphae suspension at initial concentration of ca. 8×10^4 CFU mL^{-1} were mixed with 2 ml curcumin solution (100 μM) to make a final volume and concentration of 4 ml and 50 μM curcumin respectively in a 35 mm × 10 mm petri dish. A set of 40 petri dishes each for conidia and hyphae, divided into five groups of eight, were prepared and kept in a refrigerator (4 °C) for at least 30 min, and then one by one removed from the refrigerator. Specific temperatures for each group (15 °C, 22 °C (room temperature), 25 °C, 35 °C and 45 °C) were attained by allowing dishes to warm on the bench (for those at or below room temperature) or by heating on a heating block (TDB-1 Thermoline Scientific NSW, Australia). After desired temperatures were attained, illumination was performed using 60 J cm^{-2} light dose with constant stirring (as previously described). For each temperature group, four petri dishes were illuminated (PS+/L+) while the remaining four control petri dishes were directly inoculated into growth media without illumination (PS+/L-). Temperature was monitored using a digital thermometer (Comark C26®, ComarkUSA). Conidia/hyphae concentrations after illumination were determined using CFU as described previously.

2.6. Effect of A. flavus photodegradation on aflatoxin B_1 accumulation in maize kernels

The *in vivo* experiment was conducted on intact maize kernels. Inoculation and treatment of maize was done according to the procedure explained previously (Temba et al., 2016a,b). In brief, maize kernels, sterilized by autoclaving were inoculated with *A. flavus* conidia and divided into four groups. Each group, with ten replicates of about 5 g, was subjected to specific treatments combining the photosensitiser curcumin (PS) and light (L): photosensitization group with photosensitiser plus light (PS+/L+), photosensitiser treatment group with photosensitiser only (PS+/L-), light treatment group without photosensitiser (PS−/L+) and negative control (P−/L-). For each single treatment, 5 mL of the photosensitiser (50 μM) was added to the 5 g kernels and light dose used was 60 J cm^{-2}. Treated kernels, at average initial moisture content of 18% were then incubated at 26 °C for 10 days under moist condition. Sample extraction for aflatoxin B_1 analysis was done using a mixture of acetonitrile/Milli Q® water/formic acid at a ratio of 790/200/10 according to a method by Sulyok et al. (2006) and aflatoxin B_1 level was analysed by LC-MS/MS (Shimadzu® UHPLC Nexera, and Shimadzu® 8050) (Shar et al., 2016). The analysis was conducted by using aflatoxin B_1 standards obtained from Sigma–Aldrich (Sigma-Aldrich Chemie B·V., Zwijndrecht, Netherlands). The limit of quantification for the method was 0.2 μg/kg.

2.7. Data processing and analysis

The data generated were processed and analysed using Microsoft excel® 2010. Where required, further analysis was conducted using STATISTICA® computer program. In comparing variable, statistical significance was considered when p-value was < 0.05.

3. Results

3.1. Photoinactivation of conidia and hyphae under varied pH and temperature

Treating conidia and hyphae of *A. flavus* under different pH conditions resulted in varied levels of photoinactivation as indicated in Fig. 1 and Fig. 2 respectively. The average colony counts in the non-illuminated control conidia (PS+/L-) at the pH levels of 1.5, 3.0, 5.5, 7.0 and 9.0 was 4.3 log CFU ml^{-1} (Fig. 1). The difference between the different pH levels (1.5–9.0) was not statistically significant. However, increasing the pH to 12.5 resulted in total inactivation of the conidia both in treated and untreated groups. The average colony counts for the illuminated conidia (PS+/L+) was 1.9 log CFU ml^{-1} for pH levels between 1.5 and 9.0, again with no significant difference between different pH treatments. The difference between illuminated (PS+/L+) and non-illuminated (PS+/L-) treatments demonstrated a reduction of colony count greater than 2 magnitude of log and was statistically significant (Fig. 1). Similarly the average colony count from the illuminated (PS+/L+) hyphae was 3.2 log CFU ml^{-1} compared to only 2.0 log CFU ml^{-1} for the illuminated (PS+/L+) set for pH levels between 1.5 and 9.0 (Fig. 2). This difference between illuminated (PS+/L+) and non-illuminated (PS+/L-) hyphae was statistically significant. Again at pH 12.5, almost complete inactivation of the hyphae was observed in both PS+/L+ and non-illuminated PS+/L-groups.

The resulting effects of varying the fluid temperature during photosensitization are presented in Fig. 3 and Fig. 4. In the control conidia, (PS+/L-) the average colony count was 4.1 log CFU ml^{-1}, and there was no statistical difference between temperature levels of 15 °C, 22 °C, 25 °C, 35 °C and 45 °C (Fig. 3). In contrast the average colony count in the illuminated conidia group (PS+/L+) was 1.7 log CFU ml^{-1}, a greater than 2 magnitude of log reduction when compared to the control (PS+/L-) group with the difference being statistically significant. For the illuminated PS+/L+ group an increased efficiency was observed at 45 °C when compared to lower temperatures.

The average colony count from control (non-illuminated) hyphae (PS+/L-) was 3.1 log CFU ml^{-1} (Fig. 4), and there was no statistical difference between subgroups at temperatures of 15 °C, 22 °C, 25 °C, 35 °C and 45 °C. However the average colony count from the illuminated hyphae group (PS/L+) was 1.5 log CFU ml^{-1}, a greater than 2 magnitude of log reduction when compared to the control PS+/L-group and the difference compared to the non-illuminated group was significant. Average colony count from the hyphae photosensitized at 45 °C was 0.7 log CFU ml^{-1} which was significantly lower than subgroups treated at temperatures between 15 and 35 °C (Fig. 4).

3.2. Effects of photoinactivation of Aspergillus flavus on aflatoxin production in maize kernels

Treating *A. flavus* contaminated maize kernels by curcumin mediated photosensitization, using light dose of 60 Jcm^{-2} and curcumin concentration of 50 μM resulted in reduced production of aflatoxin B_1 after 10 days of incubation (Fig. 5). The average aflatoxin B_1 in negative control (PS−/L-), photosensitiser only (PS+/L-) and light only (PS−/L+) treatment groups were 305.9 μg/kg, 317.4 μg/kg and 260.9 μg/kg respectively and there was no statistically significant difference between the three groups. The average level of the toxin in the photosensitization treatment with photosensitiser plus light (PS+/L+) was 84.2 μg/kg, which was significantly lower than the negative control group.

4. Discussion

The results from this study show curcumin-mediated photosensitization a potential method for reducing post-harvest contamination of aflatoxin B_1 in foods. The efficacy of the photosensitization was further demonstrated to be consistent across a range of pH and temperature conditions, establishing the versatility of this procedure to accommodate a range of working temperature and pH. *Aspergillus flavus*, a source of the potent carcinogenic aflatoxin, is of serious concern to food and feed safety globally. Despite the interventions of good agricultural practices, *A. flavus* infection in crops and contamination in commodities continues to occur, and hence there is a need for effective and safe decontamination procedures particularly in developing

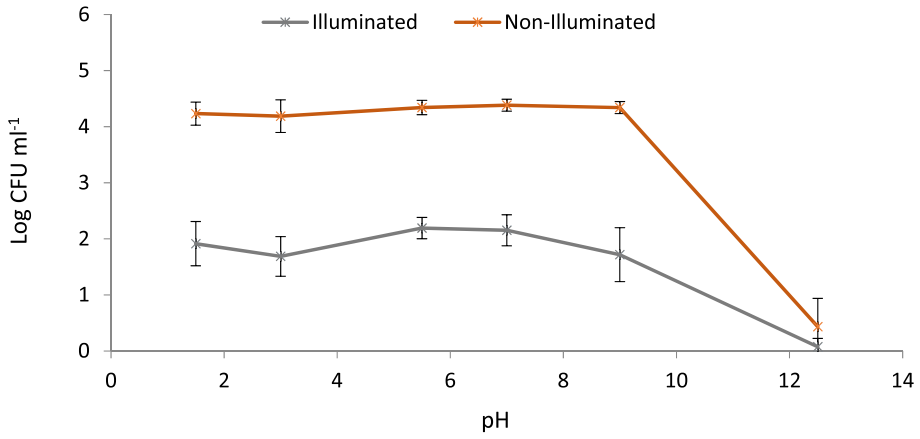

Fig. 1. Photoinactivation of *Aspergillus flavus* conidia mediated by curcumin at different pH levels with and without light illumination.

countries where agricultural practices are not as well controlled The possible integration of a locally applicable method such as photosensitization offers considerable potential in Tanzania and other developing countries.

The findings from this study show photosensitization as a promising potential interventions in reducing mycotoxin risks to consumers. The use of photosensitization mediated by natural plant products as a photo-dye for controlling microbial contamination in foods has evolved as a clean green, yet highly effective food sanitation technology (Luksiene et al., 2010; Luksiene et al., 2005; Zerdin et al., 2009). It has been shown curcumin possesses strong antimicrobial activities (De et al., 2009; Mun et al., 2013) including phototoxic activity against fungi and other microorganisms both *in vivo* and *in vitro* (Al-Asmari et al., 2017; Dovigo et al., 2011a; Dovigo et al., 2013; L. N. Dovigo et al., 2011b; Temba et al., 2016a,b). This compound has also been indicated to have inhibitory effects in broth cultures against *A. parasiticus,* by reducing its growth, production of aflatoxin B_1, and expression of aflatoxin pathway genes (Jahanshiri et al., 2012).

Our previous study showed the efficiency of curcumin mediated photoinactivation of *A. flavus* was facilitated by dye concentration and light dose (Temba et al., 2016a,b). However, the application of the technique to inhibit aflatoxin production by *A. flavus* has not previously been established. This study investigated whether the efficiency of curcumin mediated photodegradation of *A. flavus* conidia and hyphae is affected by environmental conditions, namely pH and temperature. We also investigated effects of *in vivo* photoinactivation of conidia on subsequent production of aflatoxin B_1 in experimentally inoculated maize kernels. Depending on different real-life settings, variation in pH might be a common experience in photosensitization applications such as during initial processing for maize grain milling. Previous studies have indicated pH may be an influencing factor upon efficiency of microbial photoinactivation. For instance efficiency of *Escherichia coli* K12 photocatalytic inactivation by titanium oxide was found to be favoured by reducing the pH from 7.0 to 4.0 (Rincón and Pulgarin, 2004). The efficiency of 461 and 521 nm light emitting diodes in enhancing antibacterial effect against selected foodborne pathogens was also affected by pH level (Ghate et al., 2015). However, in the current study results indicated that, across the tested range, pH level did not influence the efficiency of photosensitization. This means that if the technique was adopted at home or small scale maize processing, pH variation which might result from process variables like using treated water or applying food additives will not likely affect the efficiency of photosensitization.

The current investigation also reports a significant increase of photoinactivation efficiency to both *A. flavus* hyphae and conidia by increasing temperature from 35 °C to 45 °C. The efficiency was not changed between 15 °C and 35 °C. Temperature has been considered an important parameter in different microbial photoinactivation studies (Alves et al., 2011) with temperature considered to affect the kinetics of singlet oxygen generation and activities (Ehrenberg et al., 1998). The range of temperature tested in our study is ideally practical in both domestic and small-scale processing and represent temperatures at

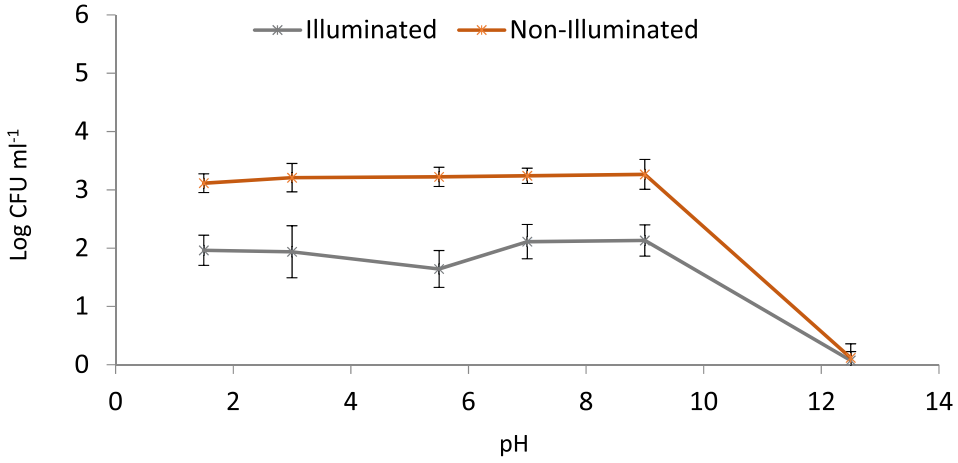

Fig. 2. Photoinactivation of *Aspergillus flavus* hyphae mediated by curcumin at different pH levels with and without light illumination.

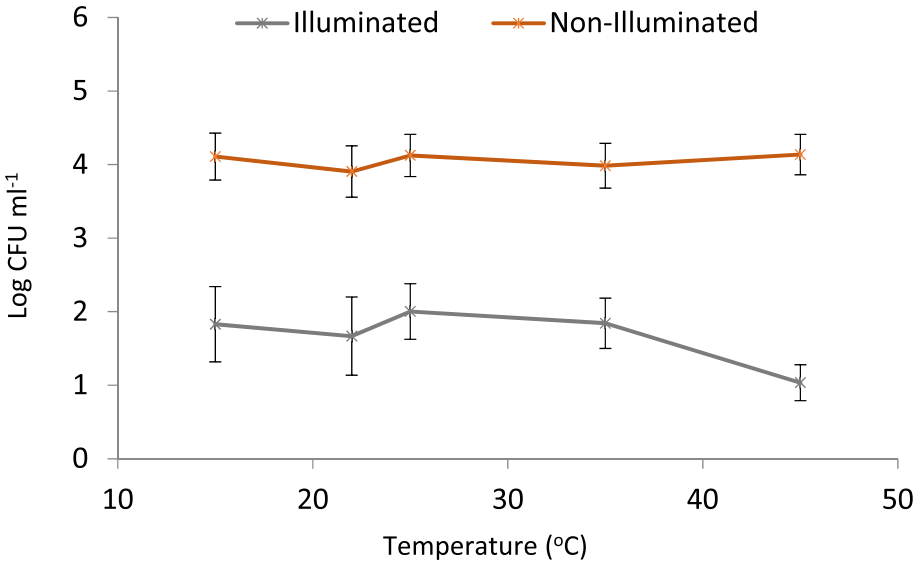

Fig. 3. Photoinactivation of *A. flavus* conidia mediated by curcumin at different temperature levels with and without light illumination.

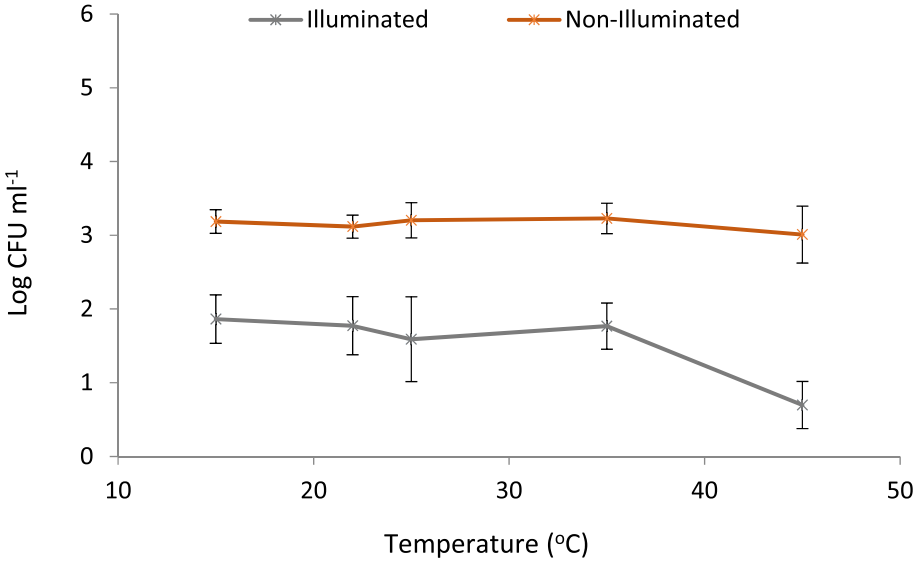

Fig. 4. Photoinactivation of *Aspergillus flavus* hyphae mediated by curcumin at different temperature levels.

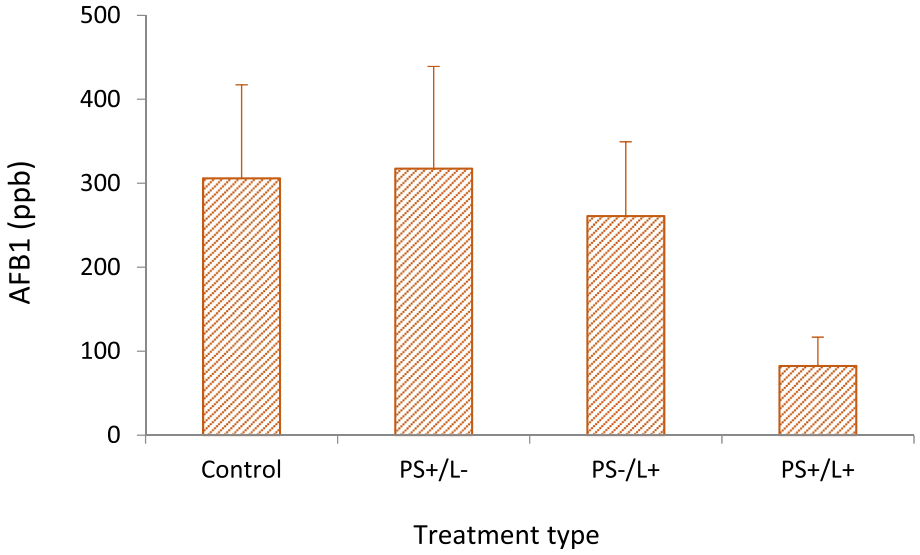

Fig. 5. Effect of curcumin mediated photosensitization of *Aspergillus flavus* inoculated maize kernels in comparison to light, photosensitiser and negative control. PS+/L- = treated with photosensitiser alone, PS−/L+ = treated with light alone, PS+/L+ = treated with both photosensitiser and light.

which food products like maize and peanuts kernels can be treated without getting "cooked". The fact that conidia and hyphae inactivation efficiency was consistent between 15 °C and 35 °C and increases at 45 °C suggests wide range application of the technique is possible across tropical and subtropical regions where the aflatoxin problem is prominent.

Measurement of aflatoxin levels in this study has further demonstrated that aflatoxin B_1 accumulation in photosensitization treated kernels is reduced to about half that formed in untreated kernels. This would at least in part be attributable to reduced number of viable fungal units leading to reduced fungal biomass, which has previously been reported to correlate with aflatoxin concentration (Mideros et al., 2009). This study thus represents the first application of curcumin for photodynamic degradation of *A. flavus* and the associated reduction of formed aflatoxin B_1. The amount of aflatoxin B_1 formed in samples treated with light only tended to be lower (although not statistically different) than that formed in samples treated with either curcumin only or in the negative control (Fig. 5). This may indicate some action of light alone on aflatoxin B_1 or its formation mechanism in the fungi. Consistent with this, light, in both UV and visible regions, has previously been reported to degrade aflatoxins and impair the fungal cells integrity (Mitoraj et al., 2007; Temba et al., 2016a,b).

The specific mechanism underlying curcumin-mediated photodynamic degradation of *A. flavus* conidia has not been reported, but the process seems to proceed through reactive oxygen species that result from the reaction between excited states of the dye molecules and oxygen molecules (Priyadarsini et al., 2003). Tonnesen et al. (1986) demonstrated curcumin molecules undergo photodegradation when exposed to UV or visible light both in solution and solid state with the formation of various products including a major cyclisation product. By itself, curcumin has antifungal activity, mediated by changing the cell membrane integrity and affect membrane-bound proteins indirectly (Lee and Lee, 2014).

5. Conclusion

For the first time, this study reports that reducing *A. flavus* conidia on maize kernels using natural dye mediated photosensitization also successfully impacts on the production of aflatoxin B_1. The study also reports that both conidia and hyphae of the fungi are inactivated almost equally under conditions of pH and temperature that correspond to normal environmental and food production ranges. These findings provide a promising technique for reducing potential aflatoxin contamination in food and feed. On farm cultivation of *C. longa* and production of extracts to treat during sun drying could help reduce *A. flavus* conidia. The technique can also be applied in food processing such as mills to reduce fungal load and enable a longer storage time for the resulting products without succumbing to high levels of mycotoxins. The kernel wetting with water which is an initial step of the typical processing of dry grains to flour, practiced mostly in tropical African countries provides ideal intervention point for the described technology.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2018.12.013.

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