Heliyon 5 (2019) e01776

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.heliyon.com

Nanostructured chitosan/propolis formulations: characterization and effect on the growth of *Aspergillus flavus* and production of aflatoxins

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ARTICLE INFO

Keywords:
Food safety
Food science
Food technology
Nanotechnology

ABSTRACT

A great diversity of agricultural products is susceptible to contamination caused by *Aspergillus flavus*. To reduce fungal contamination, the application of natural products has been proposed, including chitosan and propolis, due to its broad and recognized antimicrobial activity on several microorganisms. Currently, the application of nanotechnology allows for a greater activity to be more reactive and efficient. The objectives of this research were to characterize by TEM and Z potential some of the studied nanoparticles and to determine the *in vitro* antifungal activity of the formulations and the production of aflatoxins of the treated fungus. For this, individual treatments and different nanoformulations were elaborated by varying the percentage of the components such as chitosan solution, chitosan nanoparticles, an extract of propolis, nanoparticles of propolis, glycerol and canola oil. The final concentrations of the formulations were of 20%, 30% and 40% and the control consisted of Czapeck-dox agar medium. TEM micrographies showed a spherical morphology in a range of 2.3–3.0 nm with values of Z potential from 18.5 to 116.2 nm. Compared to the untreated fungus, the highest effect was seen in the parameter of spore germination, since inhibition was of c. a. 97% corresponding to the formulation containing chitosan + propolis nanoparticles + chitosan nanoparticles + propolis extract at the highest concentration of 40%. At this same concentration, the production of aflatoxins was 100% inhibited with the treatment with chitosan at 1%. Since these results are under carefully controlled conditions, further research should be extended to different fruit and vegetables affected by this fungus.

1. Introduction

Aspergillus flavus Link is the most common species associated with aflatoxin biosynthesis, which exposure can lead to serious human health damage. To date, the contamination of food by aflatoxins is a significant food safety issue since its presence has been detected in recently harvested and processed agricultural commodities (Battilani et al., 2008). According to Schmale and Munkvold (2018) the economic impact of mycotoxins comprises yield losses, crop values reduction, losses in animal productivity and additional costs due to related-health problems in humans.

Aflatoxins, as one of the mycotoxins, are secondary metabolites produced mainly by ascomycetous fungi including *Aspergillus* family, which are naturally occurring contaminants, principally in dried fruit, grains and cereals, and in legumes and fruit (Sanzani et al., 2016: Plascencia-Jatomea et al., 2016).

As reported by Marín et al. (2008) and Varga et al. (2010) the most employed antifungal alternative for controlling various species of *Aspergillus* including *A. flavus* is the chemically synthesized pesticides with a great variety of fungicides such as benzimidazoles and aromatic hydrocarbons, among others, and their combinations; however, the continuous application of these products increases the risk of toxic residues in the agricultural product and environment.

In addition, due to the aflatoxins and the chemicals are frequently

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https://doi.org/10.1016/j.heliyon.2019.e01776
Received 11 February 2019; Received in revised form 27 March 2019; Accepted 16 May 2019

perceived to present side effects to human health, it is necessarily important to develop ways of reducing their contamination by other non-chemical alternatives. Thus, natural compounds active formulations have become increasingly more important as a means to deliver the agricultural commodities to the consumer, in safe conditions. On this, there are various recognized plant and animal biodegradable derivatives whose antimicrobial function has been extensively reported (Ramos-García et al., 2010).

Currently, there exists a vast literature that includes the compound chitosan (poly-D-glucosamine) which is the result of extensive deacetylation of the polymer chitin (N-acetyl chitosan) as an effective antimicrobial compound. Its barrier effects, antimicrobial characteristics and mode of action against a wide range of plant pathogenic fungi and bacteria, and on foodborne pathogens is well recognized (Rodríguez-Pedroso et al., 2009; Romanazzi et al., 2017). On this, its fungicidal properties include fungi belonging to the families Mucoraceae (*Rhizopus stolonifer*), Sclerotinicaeae (*Botrytis cinerea*, *Sclerotinia sclerotiorum*), Trichocomaceae (*Penicillium digitatum*, *P. expansum*), and Pleosporaceae (*Alternaria alternata*), among others (Gutiérrez-Martínez et al., 2017).

As for the propolis or bee glue, which is a resinous mixture produced by honey bees, it is also reported to present effective antimicrobial characteristics against *Pseudomona aeruginosa, Salmonella typhi, Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Enterococcus* sp. and *Candida* spp. (Sforcin, 2016; Zabaiou et al., 2017). Overall its composition consists of wax (30%), resins (50%), balms (10%) essential oils and aromatics (5%), pollen, polyphenols, flavonoids and active components (5%).

The incorporation of these two outlined antimicrobial compounds into formulations can be an effective method to reduce microorganisms, rather than the direct addition of the component to food, because it can gradually migrate from the film or coating onto the food surface (Elsabee and Abdou, 2013). Furthermore, the study of food conservation and control of microorganisms that cause decay and disease, through the design of new materials (nanostructured formulations) has been noteworthy (Sotelo et al., 2015; Sotelo-Boyás et al., 2016).

For instance, chitosan in combinations with other compounds, and integrated into nanoformulations, that contained antimicrobial compounds such as thyme and lime essential oils, ultimately resulted in the significant control of various phytopathogenic and food-borne bacteria (Sotelo-Boyás et al., 2015; 2017a, b).

The objectives of this study were then: 1) to characterize morphologically and physically the nanostructured chitosan formulations with propolis used at 40%, 2) to evaluate the *in vitro* fungicidal activity of the nanoformulations individually or combined with different antimicrobial compounds on *A. flavus* and 3) to determine the aflatoxins production of the treated fungus.

2. Materials and methods

2.1. Fungal strain

The fungus *A. flavus* was obtained from the fungal collection of Dr. Juan C. del Río-García at the Faculty of Biological Sciences, Universidad Nacional Autónoma de México. The strain was activated on maize corns where mycelia and conidia were collected and incubated on Czapeck-dox agar medium at 20 °C.

2.2. Materials

Medium molecular weight chitosan (Sigma-Aldrich, CAS: 9012-76-4); deacetylation degree 75–85%) was used for the synthesis of nanoparticles. Glycerol was bought to J.T.Baker®. The ethanolic extract of propolis 30% was obtained from Remedios Herbolarios Rosa Elena Dueñas, S.A de C. V. and glacial acetic acid and methanol were purchased to Fermont Chemicals Inc.

2.3. Chitosan preparation

Chitosan concentration of 1.0% was prepared by adding the equal amount (w/v 1:100) of acetic acid to chitosan. The chitosan-acetic acid mixture was added to the total volume of distilled water and stirred overnight at room temperature. The solution was adjusted to pH 5.5 with 1N NaOH solution. Then 0.1 ml of Tween 80 was added.

2.4. Chitosan and propolis nanoparticles preparation

Chitosan nanoparticles were synthesized according to the methodology proposed by Correa-Pacheco et al. (2017). Medium molecular chitosan solution at a concentration of 0.05% (w/v) was dissolved in glacial acetic acid (1% v/v) and distilled water. 2.5 ml of this chitosan solution was dissolved in methanol (40 ml) by using a peristaltic pump (Bio-Rad, EP-1 Econo Pump) under moderate stirring. The obtained solution was placed in a rotary evaporator (Rotary Evaporator RE 300, BM 500 Water Bath, Yamato CF 300) at 40 °C and 50 rpm. The final volume of nanoparticles was 2 ml.

For propolis nanoparticles, a liquid extract of propolis 30% was dissolved in methanol (40%) to obtain a final concentration of 0.6%. The similar methodology used for chitosan nanoparticles preparation was followed.

2.5. Nanoformulations preparation

Different formulations were elaborated based on Correa-Pacheco et al. (2017) methodology by varying the percentage of the components such as chitosan solution, chitosan nanoparticles, extract of propolis and nanoparticles of propolis (Table 1). All formulations contained glycerol at 0.3% (J.T.Baker®) and canola oil at 0.1%. The final concentrations of the nanoparticles were of 20%, 30%, and 40%. Control consisted in Czapeck-dox agar medium.

Table 1
Nanoparticles formulations: percentage of components.

Formulations	Chitosan solution	Propolis nanoparticles	Chitosan nanoparticles	Propolis extract
20% nanoparticles				
A1	99.7	-	-	-
A2	79.7	20	-	-
A3	79.7	-	20	-
A4	99.1	-	-	0.6
A5	79.7	10	10	-
A6	79.1	10	10	0.6
A7	79.1	20	-	0.6
A8	79.1	-	20	0.6
A9	Czapeck-dox			
30% nanoparticles				
B1	99.7	-	-	-
B2	69.7	30	-	-
B3	69.7	-	30	-
B4	99.1	-	-	0.6
B5	69.7	15	15	-
B6	69.1	15	15	0.6
B7	67.1	30	-	0.6
B8	67.1	-	30	0.6
B9	Czapeck-dox			
40% nanoparticles				
C1	99.7	-	-	-
C2	59.7	40	-	-
C3	59.7	-	40	-
C4	99.1	-	-	0.6
C5	59.7	20	20	-
C6	59.1	20	20	0.6
C7	59.1	40	-	0.6
C8	59.1	-	40	0.6
C9	Czapeck-dox			

Fig. 1. Application of the formulations on Petri plates.

2.6. Application of formulations

After Czapeck–dox culture medium solidification, 1.0 ml of each individual and formulation treatment were uniformly dispersed on Petri plates (5.0 cm of diameter) (Fig. 1).

2.7. Variables evaluated

2.7.1. TEM morphology and Z potential measurement

The morphology was observed by Transmission Electronic Microscopy (TEM) (JEOL-JEM 2010) only on the following formulations: nanoparticles of chitosan used as control (C1), nanoparticles of chitosan solution + nanoparticles of propolis 40% (C2) and nanoparticles of chitosan solution + nanoparticles of chitosan 40% (C3). The particle average size (nm) was calculated using the ImageJ software. The stability of the emulsion was measured by Z potential with a Zetasizer Nano-ZS90 (Malvern Instruments).

2.7.2. Effect of formulations on A. flavus mycelia growth and conidia germination

After formulations were dried, 10 μl of the conidia concentration of *A. flavus* (10^5) were placed in the center of the Petri plates and incubated at 20 °C until fungi reached its maximum development (7 days). Radial mycelial growth of the fungus was measured every day in 6 Petri dishes of each treatment with a Truper vernier caliper, during 7 days of incubation. Data were evaluated as the mycelial rate of growth, and percentage inhibition.

For conidia germination, 10 ml of sterile water were added to four Petri dishes that belonged to the previous treatments, then conidia were harvested by scraping off the agar of each treatment. The number of spores ml^{-1} of the filtrate was adjusted to 10^5. Aliquots of 30 μl of the above spore suspension were placed onto six PDA disks of 20-mm diameter. Germination was stopped by adding lactophenol-safranin. One hundred observations were conducted per treatment using a Nikon ALPHAPHOT-2YS2–H optical microscope with a 40X objective. Evaluations were carried out during a given 0, 2, 4 6, 8 and 10 h incubation period and shown as percentage spore germination.

2.7.3. Aflatoxin production in the treated A. flavus

The described methodology was according to Méndez-Albores et al. (2009). Nine treatments of the highest nanoparticles concentration of 40% (Table 1) were placed onto six PDA Petri plates per treatment. After drying, the plates, together with 30μl of the spore suspension (10^5) of *A. flavus* were incubated for 15 days at 20 °C. After this period, the content of each Petri plate was mixed (laboratory blender model 51BL30. Waring, CT, USA) for 1 min with methanol and water (80/20 v/v). The mixture was filtered through a Whatman 100 filter paper grade and a portion of 10 mL was diluted with 20 mL of distilled water. The diluted preparation was filtered through a micro-fiber filter, and 10 mL were passed through the IACs (Afla B, VICAM Science Technology, Watertown, MA, USA). Subsequently, the column was washed with 10 mL of distilled water and dried with sterile air flow. The toxins were then eluted with 1 mL of HPLC grade methanol and quantified in a fluorometer VICAM Series-4EX (VICAM Source Scientific, Irvine, CA, USA) after reaction with 1 mL of 0.002% aqueous bromine. The detection limit for aflatoxins via fluorescence measurement was approximately 0.5 ng/g (AACC, 2000).

The average data of the aflatoxin content was based on 100 observations and 3 repetitions per treatment.

2.8. Statistical analysis

Treatments were arranged in a completely randomized design. Mean and standard deviations were also calculated. Data of the final mycelial, germination and content of aflatoxins were subjected to ANOVA and means comparison by Tukey test at $p \leq 0.05$.

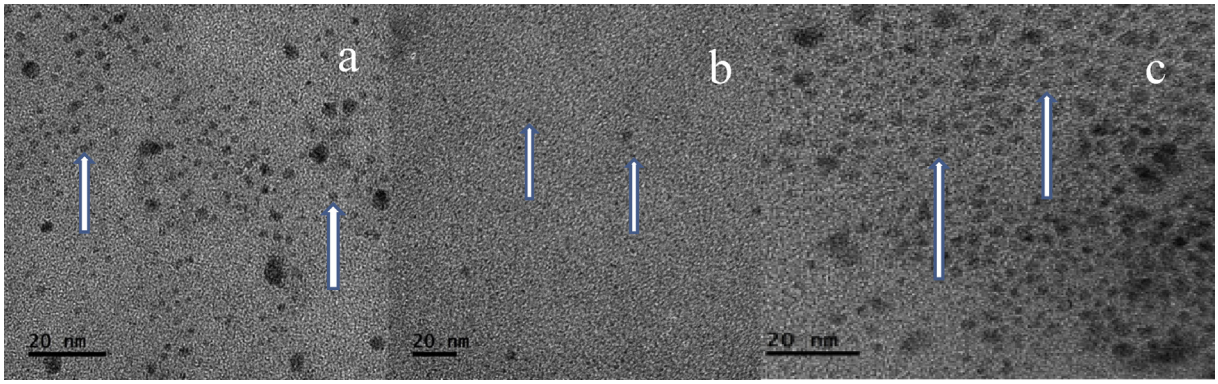

Fig. 2. TEM images of the nanoformulations at 40% concentration: a) (C1), b) (C2) and c) (C3).

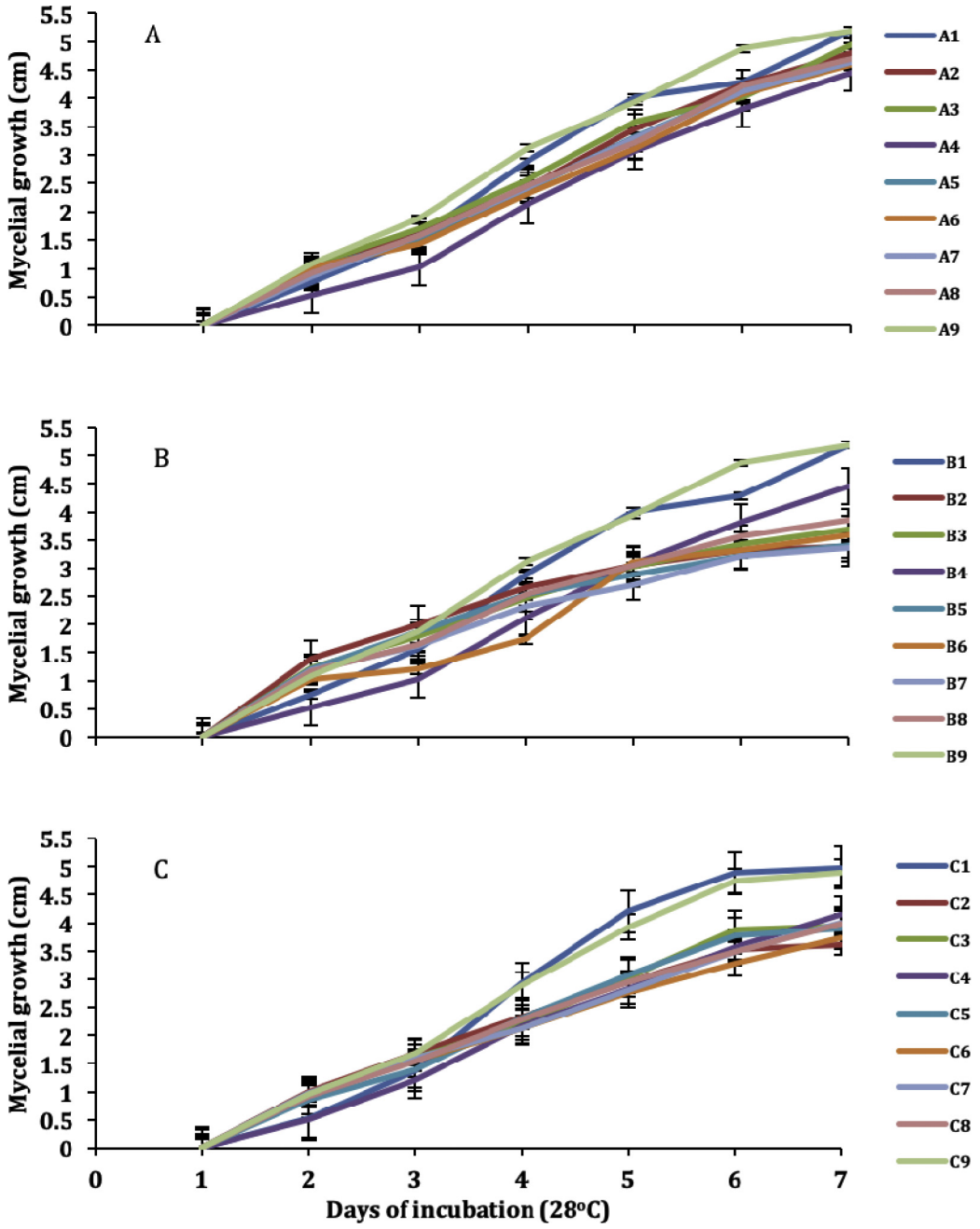

Fig. 3. Mycelia growth of *Aspergillus flavus* incubated in different nanoformulations at concentrations of 20% (A), 30% (B) and 40% (C), during a 7-day incubation period. Vertical bars indicate mean standard deviations.

3. Results

3.1. Morphology and Z potential measurement

3.1.1. Nanoparticles morphology by TEM

In the TEM images, it was observed that the morphology of the nanoparticles was spherical for all the treatments (Fig. 2a,b,c). The nanoparticles of chitosan (1.0%) evaluated as control (C1) had an average size of 3.00 ± 0.70 nm, as well as the nanoparticles of chitosan at 40% (C3) (3.00 ± 1.01 nm), while the nanoparticles of propolis at 40% (C2) had an average size of 2.33 ± 0.36 nm. In the images can be seen, that the propolis nanoparticles are well dispersed, whereas, for the nanoparticles of chitosan at 40% and control, some agglomerations were observed.

3.1.2. Z potential measurement

It was observed that as the value of the Z potential increased, the electrostatic repulsion between the particles was greater and the colloidal dispersion was more stable. The values of the Z potential were as follows: C1 = 21.3mV ± 0.06, C2 = 18.5mV ± 1.8 and C3 = 116.2mV ± 16.4. In the case of the propolis nanoparticles formulation (C2), the resin did not allow the passage of the laser beam, therefore, dilutions were done for Z potential determination.

3.2. Effect of formulations on A. flavus

3.2.1. Mycelia growth and conidia germination

In this investigation, mycelia growth and spore germination took place during each of the given incubation times (7 days and 10 h,

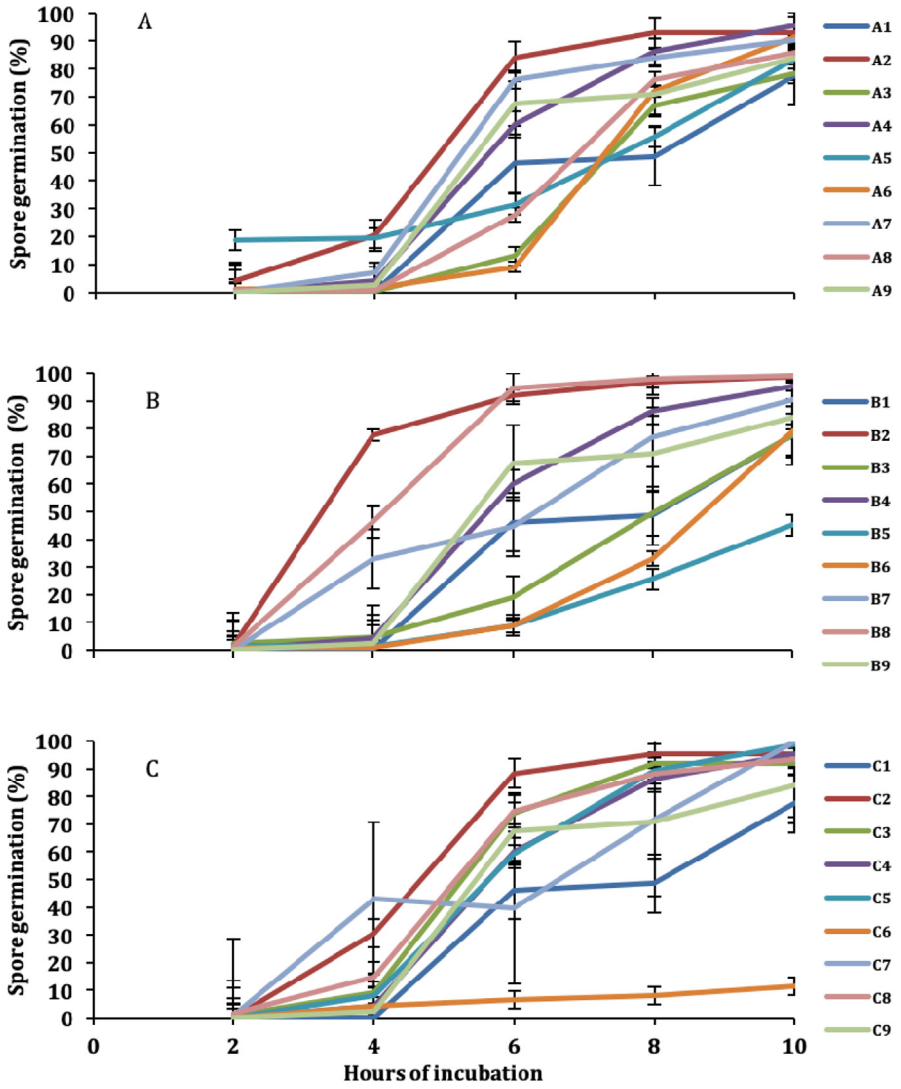

Fig. 4. Spore germination of *Aspergillus flavus* incubated in different nanoformulations at concentrations of 20% (A), 30% (B) and 40% (C), during a 10-h incubation period. Vertical bars indicate mean standard deviations.

respectively); however, with respect to the control, the growth of *A. flavus* was delayed (Figs. 3a,b,c and 4a,b,c) and significantly reduced ($p \leq 0.05$) with most of the tested formulations at the end of the given incubation periods (Table 2). There was not a clear pattern treatment-inhibition; however, the formulations containing nanoparticles of chitosan and propolis at concentrations of 30% and 40% (B2, B5, B6, and C2, respectively) showed a greater inhibition on the mycelial growth of *A. flavus* compared with the rest of the treatments, including the chitosan and propolis separately. In the case of the germination of spores, the combination of nanoparticles of chitosan, propolis and/or the extract of propolis at 40% concentration (C6, C7), presented the highest inhibition compared with the rest of the treatments.

3.2.2. *Aflatoxin production in the treated A. flavus*

With regard to the determination of total aflatoxins, all treatments dramatically inhibited its production (Table 3). For example, with chitosan alone at 1% (C1) no aflatoxin production was observed, followed by the treatments containing nanoparticles of chitosan (C3) (1.5 μg/L) and propolis extract (C4) (2.0 μg/L). The control treatment showed no inhibition, with corresponding values of 25.0 μg/L.

4. Discussion

It was observed that the nanoparticles were distributed uniformly, observing a spherical morphology. Likewise, the nanoparticles of chitosan (1.0% and 40%), presented the same size which decreased in the nanoparticles of propolis, unlike that reported by Afrouzan et al. (2012) where the size of the propolis nanoparticles was from 51 to 86 nm. This could be due to the fact that the method of making the nanoparticles was different, for example, the one used by these authors was top-down (grinding) unlike the one used in this investigation (bottom-up). Correa-Pacheco et al. (2017) and Valle (2016) reported the size of chitosan nanoparticles of 4.5 nm and 5.9 nm, respectively.

In relation to the Z potential, the obtained values for the chitosan nanoparticles coincide with Shukla et al. (2013) which reported that the Z potential of the chitosan nanoparticles made with sodium polyanion tripolyphosphate was between +20mV and +60mV. In this investigation, the propolis nanoparticles presented a lower value compared to chitosan nanoparticles 40%; which agrees with that reported by Sotelo-Boyás et al. (2015) who obtained a Z potential of +20.2 mV for the chitosan nanoparticles and +10.0 mV for the chitosan nanoparticles with lime essential oil; they argue that this could be due to the fact that propolis and lime essential oil are biologically active materials, which decrease the

Table 2
Summary of the effect of the nanostructured formulations on *Aspergillus flavus* rate of growth, and mycelial and germination inhibition at the end of the given incubation period at 20 °C.

Formulations	Rate of growth (cm/day)*	Mycelial inhibition (%)*	Germination inhibition (%)*
20% nanoparticles			
A1	0.74^{b}	0.0^{a}	22.5^{f}
A2	0.68^{b}	7.8^{c}	7.0^{b}
A3	0.70^{b}	4.7^{b}	22.0^{f}
A4	0.63^{a}	14.2^{ef}	4.5^{a}
A5	0.66^{a}	10.4^{d}	20.0^{e}
A6	0.65^{a}	12.0^{e}	10.0^{c}
A7	0.66^{a}	11.0^{d}	10.0^{c}
A8	0.67^{b}	10.0^{d}	15.0^{cd}
A9	0.74^{b}	0.0^{a}	15.3^{cd}
30% nanoparticles			
B1	0.71^{e}	0.0^{a}	22.5^{f}
B2	0.50^{a}	27.2^{e}	1.3^{ab}
B3	0.62^{c}	11.0^{b}	22.5^{fg}
B4	0.59^{b}	15.0^{c}	4.5^{c}
B5	0.50^{a}	27.2^{e}	54.8^{h}
B6	0.51^{a}	29.0^{ef}	21.0^{f}
B7	0.57^{b}	18.0^{d}	9.5^{d}
B8	0.55^{b}	20.2^{de}	0.8^{a}
B9	0.69^{d}	0.0^{a}	16.0^{e}
40% nanoparticles			
C1	0.71^{e}	0.0^{a}	22.5^{f}
C2	0.50^{a}	28.9^{e}	12.3^{d}
C3	0.55^{c}	21.2^{cd}	8.0^{cd}
C4	0.59^{d}	15.3^{b}	4.5^{ab}
C5	0.48^{a}	30.4^{ef}	1.3^{a}
C6	0.53^{b}	33.0^{f}	96.6^{h}
C7	0.57^{c}	18.1^{c}	55.5^{g}
C8	0.56^{c}	19.0^{c}	6.5^{c}
C9	0.69^{e}	0.0^{a}	16.0^{e}

*Means followed by the same letter are not significantly different ($p \leq 0.05$) determined by Tukey's multiple test.

Table 3
Total aflatoxin determination of *Aspergillus flavus* treated with different nanoformulations at 40% concentration.

Formulations	Total aflatoxins microgram/L
C1	0.0^{a}
C2	2.7^{ab}
C3	1.5^{ab}
C4	2.0^{ab}
C5	2.8^{ab}
C6	2.8^{ab}
C7	2.6^{ab}
C8	2.5^{ab}
C9	25.0^{c}

*Means followed by the same letter are not significantly different ($p \leq 0.05$) determined by Tukey's multiple test.

amount of -NH3 + ions present in chitosan in its protonated form, due to a possible interaction between chitosan and the material biologically active.

In this study, the results showed a very notable fungal growth decrease with the combination of the nanoparticles of chitosan, and nanoparticles of propolis at 30%, and the aforementioned treatments with propolis extract at 40% concentration, which clearly indicates the synergic effect among the components of the formulation. In previous reports, the synergistic activity of the combination of chitosan and propolis extract has been highlighted. Torlak and Sert (2013) reported that the antimicrobial activity of chitosan-based formulations improved when the propolis extract was added. When applying them, the growth of the bacteria was 3 log CFU, while when combined with propolis at a concentration of 10%, it was 1 log CFU. Likewise, Mattiuz et al. (2015) reported that propolis (7:3 v/v) reduced up to 75% the development of the phytopathogenic fungus *Diplodia seriata*, but when using the combination of chitosan and propolis (1/1 w/v) the inhibition was 100%.

Propolis is constituted by a great variety of chemical compounds, such as resins, aromatic balms, waxes, essential oils, pollen grains and flavonoids (Grange and Davey, 1990). In general, activity against microorganisms is related to the synergistic effect of flavonoids rather than to the action of each separately like galangina and pinocembrina; the action of these compounds can disorganize the cytoplasm, wall, and cell membrane of the microorganisms and inhibit their protein synthesis, moreover, it could be acting on the inhibition of DNA replication (Talero, 2014). On this, Cushnie and Lamb (2005) investigated the effect of galangin on cytoplasmic integrity in *S. aureus* by measuring internal potassium loss and survival, obtaining loss of up to 20% of this element and a decrease of up to 60 times of CFU. They suggest that galangin is the compound that affected the membrane, directly and indirectly, causing damage to the cell wall and osmotic lysis.

On the other hand, in this research chitosan applied alone, did not give the expected fungicidal effects as it has been shown in many different fungi. On the subject, various authors have reported that the antimicrobial effect of this natural compound may be due, among others, to the pH of the formulation, the molecular weight, the concentration, the degree of deacetylation and the type of microorganism evaluated (Bautista-Baños et al., 2006, 2017; Hosseinnejad and Jafari, 2016).

The results of this investigation agree with that reported by Juárez-Morales et al. (2017) since they also demonstrated experimentally that chitosan is able to adsorb the aflatoxin AfB1 by interacting the positive charges of the amino group of chitosan, with the negative charges of the oxygen atoms of the aflatoxins. These authors also confirmed that chitosan is a candidate to detect aflatoxins AfB1 and AfM1. Klich (2007) mentioned that chitosan acts by chelation of zinc in the inhibition of aflatoxins, which agrees with that reported by Barkai-Golan (2008) where they mention that the zinc present in the medium stimulates the production of aflatoxins in *A. flavus* and *A. parasiticus*.

Contrary to our results obtained with nanoparticles of propolis, Hashem et al. (2012) reported a total inhibition in the production of aflatoxins on *A. parasiticus* by applying the same 0.6% concentration of propolis extract, although in this case, the origin of the extract was from Saudi Arabia. These authors mentioned that the mechanism of propolis activity depends on the synergy between the flavonoids, the phenolic acids in propolis and their interference with aflatoxin biosynthesis, and they explained that there is a genetic correlation between the production of conidia and the secondary metabolites.

All the obtained values were below than those obtained with the control treatment (25 ppb). According to the Mexican Official Standard NOM-188-SSA1-2002, the maximum limit of total aflatoxins is 20 ppb.

5. Conclusions

In this study, we have identified that the components in the formulation based-chitosan combined with nanoparticles of chitosan and propolis, and extract of propolis, at the highest concentration of 40%, exerted a notable inhibition on the spore germination and principally, on the aflatoxin production of *A. flavus*. Nevertheless, since these results are under carefully controlled conditions, further research should be extended to *in vivo* proposal on different agricultural commodities affected by this fungus.

Declarations

Author contribution statement

Monica Cortes-Higareda: Performed the experiments; Analyzed and interpreted the data.

Margarita de Lorena Ramos-García Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Zormy Nacary Correa-Pacheco: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Juan Carlos Del Rio Garcia: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Silvia Bautista-Banos: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Funding statement

This work was supported by Instituto Politécnico Nacional through the Secretary of Research and Postgraduates (project 20180091 'Nanostructured coatings with antifungal properties on aflatoxins fungi-promoting, for their application in agricultural commodities') and Universidad Autónoma del Estado de Morelos.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

Acknowledgements

The authors acknowledge the experimental support of the National Polytechnic Institute-Center for Nanosciences and Micro and Nanotechnologies.

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