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Antifungal efficacy of chitosan nanoparticles against phytopathogenic fungi and inhibition of zearalenone production by *Fusarium graminearum*

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Abstract

Chitosan (COS) is a natural safe biopolymer that received great attention in agriculture, food, biomedical, pharmaceutical and environmental industries because their biocompatible, biodegradable, non-toxic and non-allergenic natures. The aims of the current study were to synthesize and characterize chitosan nanoparticles (COS-NPs), to evaluate their antifungal activity against phytopathogenic fungi and inhibition of zearalenone (ZEN) production by *Fusarium graminearum*. The results revealed that the deacetylation degree of COS was 86.9 0.44 %, the average of molar mass was 171.41 ± 0.29 g/mol, molecular weight was 244 ± 7 kDa and the concentration of free amino groups was 0.05 ± 0.019 mol L^{-1}. COS-NPs showed the nanorod form with rough nature and particle size was around 180 nm. COS-NPs showed an excellent antifungal activity against *Alternaria tenuis*, *Aspergillus niger, A. flavus, Baeuvaria bassiana*, *Fusarium graminearum, Fusarium oxysporum*, *Penicillium* sp. and *Sclerotium rolfsii* in dose dependent manner. At a concentration of 800 ppm, it inhibits ZEN production by *Fusarium graminearum*. It could be concluded that COS-NPs are promise candidate as safe antifungal capable for the prevention of ZEN production.

Keywords: *chitosan, chitosan nanoparticles, antifungal activity, phytopathogenic fungi, zearalenone*

Introduction

Fungal invasion and mycotoxin contamination are considered the most global threat to food and feed (Pitt and Hocking, 2009). Fungal invasion affect the nutritional quality, color, texture and rancidity leading to food spoilage (Magan et al., 2010). Moreover, the presence of mycotoxins in food and feed possess great hazards to humans and animal health. Zearalenone (ZEA) also called F-2 toxin is nonsteroidal estrogenic mycotoxin produced by *Fusarium* spp mainly *F. graminearum* gained the attention of researchers (Zinedine et al., 2007). ZEA is immunosuppression, neurotoxic, hepatotoxic and carcinogenic (Ben Salah-Abbès et al., 2010; Venkataramana et al., 2014). ZEA is also responsible for abortions, hypertrophy of gonads and hyperestrogenism in females (Zinedine et al., 2007). According to the International Agency for Research on Cancer (IARC, 1999), ZEA is carcinogenic in laboratory animals and classified as a Group 3 carcinogen. *F. graminearum* can adapt and grow in different environmental conditions and has the ability to produce ZEN (Bernhoft et al., 2012). For this reason, control measures and successive management of *F. graminearum* growth and toxins production are in great demand. The excessively used of synthetic fungicides induce toxicity and accumulated in the environment (Reimann and Deising, 2000). Consequently, there is a need

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for cost effective and strategies to control the growth of *F. graminearum* and ZEN production.

Chitosan (COS) is a natural polysaccharide consists of varying amounts of (1-4)-glycosidic bonds linking N-acetyl-2-amino-2-deoxy-Dglucopyranose (glucosamine) and 2-amino-2-deoxy-D-glucopyranose (N-acetyl-glucosamine) (Agrawal et al., 2010). COS is one of the most abundant natural polysaccharide biopolymers and amino polysaccharide extracted from insects is considered the best known type, crustaceans and cellular walls of fungi (Kean and Thanou, 2010). COS has attracted the attention in the area of biomedical material and it exhibited a wide variety of biological activities, such as immunostimulating effects (Jeon and Kim, 2001), antitumor activities (Santosh et al. 2012), hemostatic agent (Ruolan et al., 2010), antiallergic effects (Thanh-Sang et al., 2012), anticoagulant effects (Jianglin et al., 2011), antiinflammatorty activities (Chung et al., 2012), free radical scavenging activities (Anraku et al., 2008), hypocholesterolemic effects (Douglas et al., 1998), antimicrobial and antibacterial (Park et al., 2004; Yong et al. 2011) wound-healing effects (Jayakumar et al., 2011; Tao et al., 2012), antifungal activities (Qin et al., 2012) and antiviral activity (Hui et al., 2012). The antimicrobial activity of COS and its derivatives against most economic plant pathogens have been received considerable interest (Badawy and Rabea, 2011; Xia et al., 2011). Consequently, COS is considered as useful pesticides in the control of plant diseases to avoid the harmful effects of synthetic antimicrobial agents (Kaur et al., 2012; El-Mohamedy et al., 2014). Kaur et al. (2012) also reported that COS can inhibit fungal growth in the developmental stages such as mycelial growth, spore formation, spore viability and germination. The other important attribute of COS is associated with its fungistatic or fungicidal properties against pathogens of different types of fruits (Bautista-Baños et al., 2003). Growth of important postharvest fungi such a *Penicillium* spp, *Alternaria alternate, Colletotrichum gloeosporioides*, *Fusarium oxysporum, Rhizopus stolonifer* was inhibited on media supplemented with various concentrations of COS (Bautista-Baños et al., 2003).

However, COS has a high molecular weight and water-insolubility which make its applications are severely limited. Due to its water-insolubility, the function and applicability of COS is limited. To cope this problem, nanoparticles (NPs) formulation can provide a feasible pharmaceutical basis to enhance bioavailability and efficacy of COS (Jia, 2005). El-Denshary et al. (2015) and Abdel-Wahhab et al. (2015) reported that Chitosan nanoparticles (COS-NPs) exhibit more superior activities than COS and heightened the immune-enhancing effect, the anticancer activity and the antimicrobial activity compared to COS. Previously, Müller and Böhm (1998) suggested that NPs possess a stronger curvature of the surface compared to the large particles resulting in more dissolution pressure and a corresponding increase in saturation solubility. The aims of the present work were to synthesis and characterizes COS-NPs and to evaluate their effects on colony growth and toxin production of some phytopahogenic fungi *in-vitro*.

Material and Methods

Materials

Chitosan was purchased from Sigma-Aldrich (France). All media and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Characterization of chitosan (COS)

The degree of deacetylation (DA%) of COS was determined by the infrared spectroscopy method suggested by Brugnerotto et al. (2001) and UV spectrophotometric according Liu et al. (2006). Free amino group concentration and the average of molar mass of COS monomers were determined according to the method described by Aljawish et al. (2012). However, the molecular weight of COS was evaluated using size exclusion chromatography with multi-angle laser light scattering detectors (SEC-MALLS) according to Nguyen et al. (2009). The whole measurements were carried out in triplicate at the Food Toxicology and Contaminants Department, National Research Center, Dokki, Cairo, Egypt.

Preparation of chitosan nanoparticles (COS-NPs)

COS-NPs were prepared according to

the method described by Tang et al. (2007) and Abdel-Wahhab et al. (2015). In brief, 20 mg COS was dissolved in 40 ml of 2.0% acetic acid (v/v). A 20 ml of 0.75 mg ml^{-1} sodium tripolyphosphate was dropped slowly with stirring on COS solution and COS-NPs as a suspension were collected and stored in deionized water. The supernatant was discarded and COS-NPs were air dried for further use and analysis.

Determination of particles size and morphology of COS-NPs

The size and morphology of the prepared COS-NPs were analyzed using Nanotrac Analyzer 6Hx4Wx15D, Model-Nanotrac 150 with a measuring range of 0.8-6500 nm. The COS-NPs were then cut into pieces of various sizes and wiped with a thin gold-palladium layer by a sputter coater unit (UG-microtech, UCK field, UK) and the morphology of nanoparticles was analyzed with a Cambridge stereoscan 440 Scanning Electron Microscope (SEM, Leica, Cambridge, UK). FTIR spectra of CS and COS-NPs were determined by infrared spectrometer (FTIR) (Thermo Fisher Scientific Inc., Nico-let iS10, USA).

Test fungi

Ground nut oil seed plants were collected from the environmental farm of National Research Centre, Dokki, Cairo, Egypt. One hundred seeds were surface disinfected by soaking in 2% sodium hypochlorite for 3 min followed by 70% ethanol for 2 min then thoroughly washed in sterile water. The excess water was drained and the seeds were dried between two layers of sterilized filter papers. The dried seeds were platted on potato dextrose agar (PDA) medium at rate of 4 seeds/ dish (Foor et al., 1976) and were incubated at 27 ± 2˚C for 7 days. Fungi growing from the seeds were isolated, purified and identified as described by Barnett and Hunter (2000) and Domsch et al. (2007). All isolates were maintained on PDA slants and incubated for 7 days at 27 ± 2˚C, which served as test fungi for antifungal activity assay.

Assessment of antifungal assay

Mycelium growth inhibition

In-vitro assay for mycelium growth was performed on growth medium treated with different concentrations of COS-NPs (0.0, 100, 200, 400 and 800 mg/ kg media) against the isolated fungi. The agar plates were inoculated by a 5 mm disc of 7 day- old phytopathogenic fungi and incubated at 27 ^{O}C until the control plates fully grow. The radial inhibition was calculated in comparison with the control plate. The toxicity of the COS-NPs to the fungal growth term of percentage inhibition of mycelial growth was calculated using the following formula

% inhibition =dc- dt/dc x100

according to Singh and Tripathi (1999).

Where dc= Av increase in mycelial growth in control plates, dt= av increase in mycelial growth in COS-NPs-treated plates.

Agar well diffusion method

Different concentrations of COS-NPs were screened for the antifungal activity by agar well diffusion method with sterile cork borer of size 6.0 mm according to Bobbarala et al. (2009). Seventy two hours old cultures grown on PDA medium broth were used for inoculation of fungal species on PDA plates. A volume of 0.2 ml aliquot of inoculums was introduced to molten PDA and poured in to a petri-dish. The appropriate wells were made on agar plate after solidification and 500 µl of each concentration of COS-NPs solutions (e.g. 0, 25, 50, 75, 100 and 125 mg 100/ ml distilled water) were homogenized using an ultrasonic cleaner and filled in deep blocks. Incubation period of 4-5 days at 27 ± 2˚C was maintained for observation of antifungal activity by the measurement of the zone of inhibition of fungal growth (in mm) surrounding the well. All experiments were carried out in triplicates and the synthetic fungicide (8-hydroxy quinolin, 100 µg/ml) was used as control.

Effect of COS-NPs on Zearalenone (ZEN) production by Fusarium graminearum

Uniform discs (5 mm diameter) including mycelia and conidia of F. *graminearum* were transferred from the edge of a 7-day-old culture to petri dishes containing 15 ml PDA medium containing 800 ppm COS-NPs. Other Petri dishes were inculcated with the fungi without the

addition of COS-NPs and were used as control. The dishes were incubated for 14 days at 25 °C then all the dishes were autoclaved and the medium was used for the determination of ZEN. All assays were performed in triplicates.

Determination of Zearalenone (ZEN)

The determination of ZEN was carried out by HPLC as described by Sabater-Vilar et al. (2007) with a little modification. One and a half ml (1.5 ml) of each liquid culture was extracted with 7.5 ml of chloroform. The water phase was discarded and the chloroform was evaporated under a nitrogen stream. The obtained residue was re-dissolved in 100 µl of mobile phase and 20 µl were injected into HPLC. The mobile phase consisted of methanol/ water (65:35 v: v). A 5 ml C_{18} Luna II stainless steel column (150 x 4.6 mm; Phnomenex, The Netherlands) was connected to high precision pump (Gynkotek model 300) with a flow rate of 0.7 ml/min and controlled by Chromeleon-Gynkotec HPLC software (Softron). Fluorescence detection was performed with a FP 920 fluorescence detector (Jasco, Japan) set at 236 and 418 nm excitation and emission wavelength, respectively. The limit of quantification was estimated to be 0.04 mg ZEN/ml.

Statistical Analysis

All data were statistically analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (Duncan 1957) using statistics software package SPSS for Windows, V. 13.0 (Chicago, USA). P values ≤ 0.05 were considered as statistically significant.

Results

The results of the current study revealed that the deacetylation degree of COS was 86.9 ± 0.44 % and the average of molar mass was 171.41 ± 0.29 g/mol. The molecular weight of COS was 244 ± 7 kDa and the concentration of free amino groups was 0.05 ± 0.019 mol/L. The scanning electron microscopy (SEM) images showed the nanorod form of COS-NPs and the atomic force microscopy (AFM) topography indicated the rough nature of the surface (Fig 1). The particle size was around 180 nm and distribution in the range of 50-800 nm (Fig. 2). The FT-IR spectra revealed that COS-NPs showed a new peak appear at 1256/cm which indicate P=O stretching (Fig. 3).

The current results showed that eight

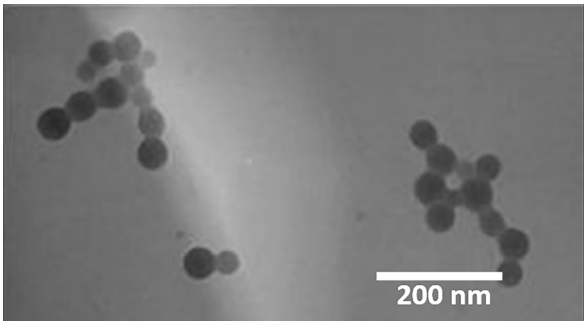

Figure 1. Scanning electron microscopy of COS-NPs.

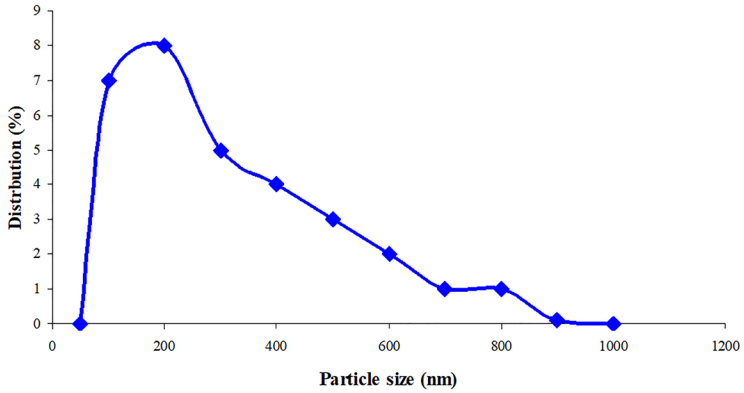

Figure 2. Particles size distribution of COS-NPs.

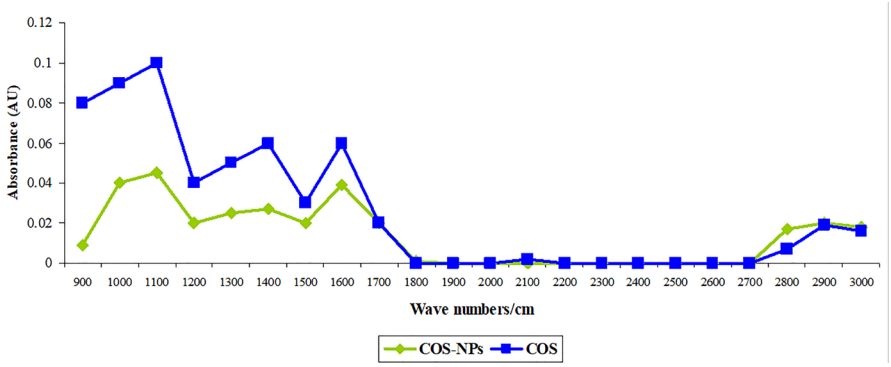

Figure 3. The FT-IR spectra analysis of COS and COS-NPs.

species were isolated from ground nut oil seeds included *Alternaria tenuis*, *Aspergillus niger, A. flavus, Baeuvaria bassiana*, *Fusarium graminearum, Fusarium oxysporum*, *Penicillium* sp. and *Sclerotium rolfsii.* The antifungal activity of COS-NPs represented as the effect of COS-NPs on the percentage inhibition of mycelial growth of some phytopathogenic fungi is presented in Table (1). The results showed a positive correlation between the concentrations of COS-NPs and colony growth, with the increasing of Cos-NPs concentration a significant increase in the growth inhibition was found for all fungi. However, the sensitivity of different species to COS-NPs was varied. The inhibition percentage of *B. bassiana* was significantly high (60.57 % inhibition mean) than the other tested fungi, followed by *F. oxysporum,* then *F. graminearum* and *Alt. tenuis* which recorded 42.02, 40.53 and 40.53% mean inhibition, respectively without any significant difference (Table 1).

The current results revealed that the percentage of mycelium growth inhibition at the higher concentration (800 ppm) was varied from 37.41% for *S. rolfsii* to 76.08 % for *B. bassiana*. These findings indicated that there are variations in tolerance to COS-NPs between different tested fungi. COS-NPs also proved to be effective and gave the most promising antifungal activity at concentration as low as 100 ppm against *F. graminearum* responsible for 42.81 % inhibition followed by *F. oxysporum* (32.11 % inhibition). Moreover, COS-NPs was quite effective against *S. rolfsii* which recorded 12.06% mean inhibition (Table 1).

There was a tendency towards increase

Table1. Antifungal activity (% inhibition) of different concentrations of Cos-NPs on the linear growth (mm) of different phytopathogenic fungi.

Fungi	Concentration (ppm)				
	0	100	200	400	800
Alternaria tenuis	0.00	20.22^{a}	54.87^{f}	60.00^{j}	67.67^{n}
Aspergillus niger	0.00	21.33^{a}	48.92^{g}	53.33^{k}	62.75^{o}
Aspergillus terreus	0.00	23.33^{b}	48.33^{g}	58.67^{j}	74.67^{c}
Baeuvaria bassiana	0.00	73.92^{c}	74.83^{c}	78.00^{q}	76.08^{c}
Fusarium graminearum	0.00	42.81^{d}	45.19^{h}	54.29^{k}	60.37^{j}
Fusarium oxysporum	0.00	32.11^{e}	48.45^{g}	62.96^{l}	66.60^{n}
Sclerotium rolfsii	0.00	0.00	2.52^{i}	20.37^{m}	37.41^{p}

Three replicates were used for each treatment. Within each raw, values superscript with the same letter are not significantly different at P ≥ 0.05 according to Duncan's multiple range test.

in the zone of inhibition rates proportional to the increase in the tested concentrations of COS-NPs (Table 2) suggesting that there were significant differences among all the tested COS-NPs concentrations. COS-NPs at higher concentration (125%) exhibited high inhibition against *F. graminearum* then *Penicillium* sp. followed by *B. bassiana* and *A. flavus*, as the zone of inhibition recorded 44.3, 42.67, 36.67 and 36.67 mm for the four fungal spp respectively. The highest value of antifungal index was observed in medium containing COS-NPs at a concentration of 800 ppm for each fungus. As a broad-spectrum fungicide, COS-NPs have been proved to be fungicidal against many pathogenic fungi.

The determination of ZEN in the medium containing 800 ppm of COS-NPs and inculcated with *F. graminearum* using HPLC revealed that

Table 2. The anti fungal activity of COS-NPs against different phytopathogenic fungi (Zone of inhibition values in mm).

Tested organisms	Concentration %						*Fungicide
	0	25	50	75	100	125	100 µg/ ml
F. graminearum	0.00	0.00	9.00^{ab}	15.33^{dg}	20.67^{h}	44.3^{i}	42.33^{A}
A. flavus	0.00	8.67^{a}	10.33^{c}	13.00^{e}	15.67^{dg}	34.67^{j}	34.00^{B}
B. bassiana	0.00	0.00	9.67^{ab}	12.67^{e}	16.33^{g}	36.67^{k}	39.33^{C}
Penicillium sp.	0.00	10.00^{b}	14.67^{d}	18.67^{f}	21.33^{h}	42.67^{l}	42.00^{A}

Positive control for fungi= 8 hydroxy quinoline sulphate. Three replicates were used for each treatment. Within each raw, values followed by the same letter are not significantly different at P ≥ 0.05 according to Duncan's multiple range test.

ZEN standard was eluted at 10.991 min (Fig. 4a) however; in the control samples, ZEN was eluted at 11.072 min (Fig. 4b). The media inculcated with *F. graminearum* and treated with 800 ppm of COS-NPs did not show any peak area at the time of ZEN elution (Fig. 4c) which support our findings of fungal inhibition and consequently the absent of ZEN in COS-NPs-treated media.

Discussion

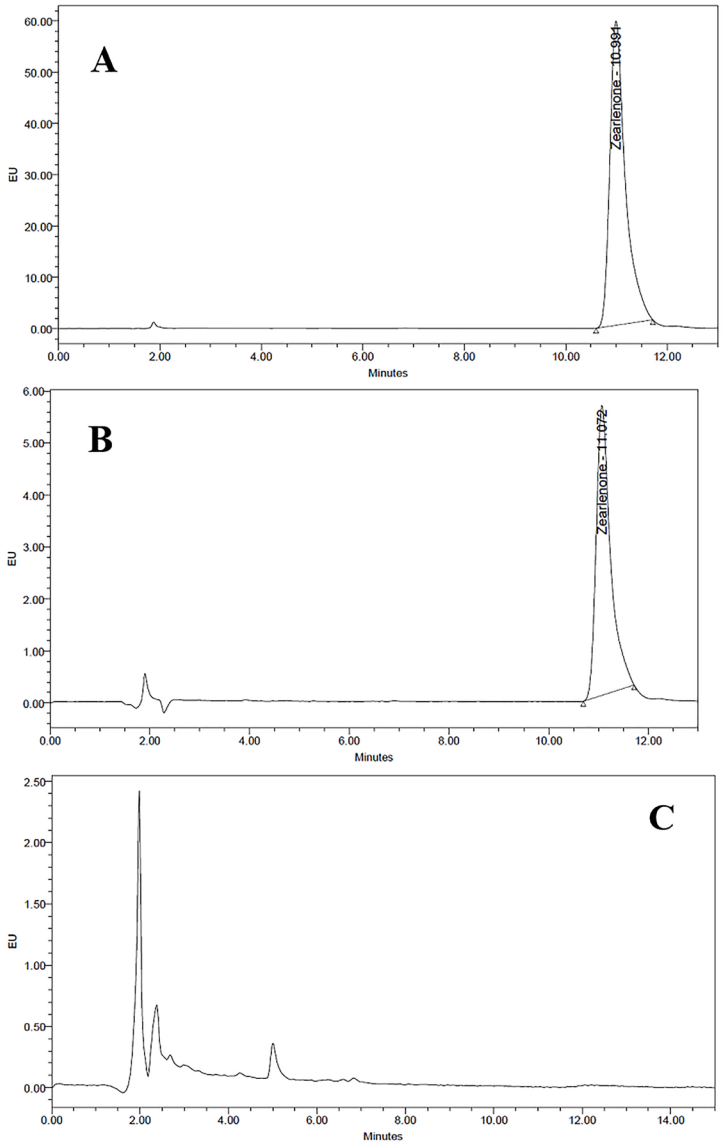

Figure 4. An HPLC chromatogram of (A) authentic standard of ZEN showing that ZEN eluted at 10.991 min, (B) control samples showing that ZEN eluted at 11.072 min and (C) COS-NPs treated samples showing the absent of ZEN.

The current results of FT-IR showed that anionic phosphate groups of sodium polyphosphate interacted with the cationic amino groups of COS which enhanced both the inter- and intramolecular interaction in COS as suggested in the recent studies (Abdel-Wahhab et al., 2015; Antoniou et al., 2015). Furthermore, the UV-Vis spectra confirmed the formation of a complex between sodium tripolyphosphate and COS. It was reported that the range of 250-350 nm is corresponding to the electronic transition involving the single pair of electrons on the sodium tripolyphosphate oxygen atom and/or phosphate group (Fahima et al., 2013). Furthermore, the appearance of the broad band around 350-450 nm suggested the formation of the complex between the oxygen and/ or phosphate groups and ammonium groups of COS. These results strongly suggested the possibility of the interaction between the cationic amino groups of COS and the anionic phosphate groups of sodium polyphosphate. The average particle size of the prepared COS-NPs was 180 nm. Several reports suggested that particles size have influence on the inhibition of fungal growth and smaller nanoparticles had stronger antifungal effect due to the diffusion of COS-NPs into the cells of fungi resulting in the inhibition of RNA and DNA synthesis, subsequently causing a direct cell death (Martinez-Gutierrez et al., 2010; Ing et al., 2012).

The current results showed that COS-NPs at higher concentration (125%) exhibited high inhibition against *F. graminearum* then *Penicillium* sp. followed by *B. bassiana* and *A. flavus* with significant difference, as the zone of inhibition were 44.3, 42.67, 36.67 and 36.67 mm respectively. According to Bautista- Baños et al. (2003), the degree of fungicidal activity has a direct relationship with the concentrations of nano-formulation. According to Kim et al. (2012), the most significant inhibition of plant pathogenic fungi was observed on PDA at 100 ppm COS-NPs. However, at the low concentration (25%) of the COS-NPs, the zone of inhibition was increased reached 8.67 and 10.00 mm for *A. flavus* and *Penicillium* sp, respectively compared to 0.0 mm in the control. In a previous work, Cu-COS-NPs at 0.12% concentration caused 70.5 and 73.5% inhibition of mycelia growth in several species included *Alternaria solani* and *Fusarium oxysporum*, respectively (Saharan et al., 2015). Moreover, Ing et al. (2012) reported that COS-NPs showed antifungal activity against *Candida albicans*, *Fusarium solani* and *Aspergillus niger*.

The results of the current study revealed also the variation in sensitivity for different fungal species to COS-NPs. The different tolerance of the tested fungi to COS-NPs may be due to the difference in fatty acid composition. However, the plasma membranes of COS-sensitive fungi were shown to have more polyunsaturated fatty acids than COS-resistant fungi (Palma-Guerrero et al., 2010). It is well known that the genetic of fungal species play an important role in COS sensitivity. According to Palma-Guerrero et al. (2010), the most sensitive fungi to COS have deletions of genes related to functions involving cell wall/cell membrane biogenesis. This group included deletions of genes that were involved the factors that affect plasma membrane fluidity such as sphingolipid and ergosterol biosynthesis which was proposed to influence cell sensitivity to COS (Galván et al., 2013). The strains with deletions of genes affect cell wall and cell membrane biogenesis and showed highly sensitive to COS support the membrane damage mechanism hypothesis (Park et al., 2008). Consequently, differences in cell membrane ergosterol, phospholipid, glycosphingolipid and membrane proteins may display some correlation with COS sensitivity (Xing et al., 2016). Moreover, the variation in fungal resistance to COS-NPs was suggested by Allan and Hadwiger (1979) who reported that fungi that have COS as one of the components of cell wall are the most resistant to the externally amended COS.

Previous reports on the mechanism of inhibitory action of nanoformulationon against microorganisms have shown that upon treatment with the nanoparticles, DNA loses its ability to replicate resulting in inactivated expression of ribosomal subunit proteins, as well as certain other cellular proteins and enzymes essential to the production of ATP (Feng et al., 2000). It has also been hypothesized that nanoparticles primarily affects the membrane-bound enzymes function, such as those in the respiratory chain (Bragg

and Rainnie, 1974). According to Saharan et al. (2015), fungi have tendency to produce different level of acids during growth resulting in acidic pH which induces the protonation of amino groups of COS leading to damage biomolecules (Brunel et al., 2013). The effect of COS as defense enzyme inducer may be another mechanism of the higher antifungal activity of COS-NPs (Borkow and Gabbay, 2005). On the other hand, the different tolerance of fungi to COS-NPs may be due to the difference in unsaturated fatty acid composition which is the key part of phospholipids in the cell membrane's lipid bilayer which represent the important factor influence the membrane stability and fluidity. Generally, the antifungal activity of COS-NPs contributes to larger surface area which enables COS-NPs to adsorb more tightly onto the surface of fungal cells and disrupt the membrane integrity (Qi et al., 2004). Moreover, COS-NPs might be able to diffuse into fungal cell and hence disrupt the synthesis of DNA as well as RNA (Ing et al., 2012), consequently induce its high antifungal activity. More important, exposure to COS-NPs resulted in the formation of impermeable layer will block the channels on the cell surface and hence prevent the transportation of the essential nutrients which are crucial for survival of microbial cells (Qin et al., 2006).

Conclusions

It could be concluded from the current study that the characterization of COS revealed that it has low molecular weight and high degree of deacetylation. This resulted in an increase of positive charges due to the free amino groups and thus facilitates the coupling process with cell membrane of fungi. COS-NPs with particles size of 180 nm showed a strong antifungal activity against the eight tested fungi. COS-NPs induced the antifungal activity through several mechanisms the disturbance of cell membrane function, disturbance DNA and RNA synthesis and block the channels on the cell membrane through the formation of impermeable layer. Consequently, COS-NPs may be promise candidate as fungicidal against seed borne pathogenic fungi

Acknowledgments

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