Application of chitosan and chitosan nanoparticles for the control of Fusarium head blight of wheat (Fusarium graminearum) in vitro and greenhouse

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\textbf{A B S T R A C T}

Fusarium head blight (FHB) disease caused by \textit{Fusarium graminearum} is one of the most important diseases of wheat in humid and warm areas. This disease significantly reduces yield as well as seed quality. The aim of this work was to evaluate the possibility of control of FHB by chitosan (CS) and chitosan nanoparticles (CS/NPs). In vitro, the application of various concentrations of CS and CS/NPs showed significant inhibition of both radial mycelial growth and number of colonies formed against \textit{F. graminearum}. The application of 1000 and 5000 ppm concentration of CS and CS/NPs produced maximum inhibition of radial mycelial growth in comparison to the control, respectively. The microscopic examination of treated \textit{F. graminearum} with the CS and CS/NPs, showed dehydration and deformation in mycelial growth and some hyphae were collapsed. The maximum percentage reduction number of colonies was observed in 5000 ppm concentration of both CS and CS/NPs. To test the effect of CS and CS/NPs on spore germination, four concentrations were used for 4 and 24 h incubation. The 24 h incubation of \textit{F. graminearum} spores with a 5000 ppm solution of CS greatly reduced the number of germinating spores. In greenhouse trials, the disease severity percentage was low when CS and CS/NPs were applied before fungus inoculation on the plants and 1000 ppm concentration. The spores of \textit{F. graminearum} germinated on the anther, hyphae penetrated into anther and colonized the palea, lemma and glume after 24 and 72 hpi, respectively. Wherease, the spikelets treated with CS and CS/NPs were infected slowly. Light microscopy and TEM observations indicated that mycelium penetrated into the cells through stomata and transited to other cells by cell wall or plasmodesmata. Mycelial growth caused conidia into cells but CS and CS/NPs prevented of it’s growth. Results showed that CS and CS/NPs could be a useful biological pesticide for controlling FHB.

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1. Introduction

Fusarium head blight (FHB) or scab is one of the main diseases of wheat in humid and warm areas of the world. Yield losses in seven epidemic years exceeded more than 1 million tons wheat in China [1] and 70 million tons barley in USA [2]. In addition to, FHB produces mycotoxins. The feeding of contaminated grains causes disorders in human and animals. In order to control FHB, chemicals have been used for a long time. The foliar application of fungicides such as tilt at anthesis might provide some protection, but chemicals destroy natural enemies of plant pathogens (antagonists) and induce pathogen populations resistant to chemicals. Thus, plant disease will outbreak with more severity. Also, they may enter the food chain and accumulate in the human body and cause diseases in it [3]. Thus, researchers have tried to develop biological pesticides with less dangers.

The antimicrobial activity of chitosan (CS) has been recently studied and it has been demonstrated that CS has the ability to inhibit and kill a wide variety of microorganisms. In addition to,
CS has a lower toxicity toward mammalian cells in comparison with chemicals [4]. Antifungal activity of CS has been observed against Penicillium digitatum, Macrophomina phaseolina, Fusarium solani, Phomopsis asparagi, Fusarium oxysporum f. sp. cubense, Colletotrichum gloeosporioides, Rhizopus stolonifer, Fusarium fujikuroi, F. oxysporum f. sp. lycopersici, Sclerotium rolfsii and Rhizoctonia solani [5–13]. There are three mechanisms proposed as the inhibition mode of CS. In the first mechanism, plasma membrane of fungi is supposed the main target of CS. The positive charge of CS enables it to interact with negatively charged phospholipid components of fungal membrane. This will increase the permeability of membrane and causes the leakage of cellular contents, which subsequently leads to cell death [14,15]. Based on the second mechanism, CS acts as a chelating agent by binding to trace elements, causing the essential nutrients unavailable for normal growth of fungi [16]. Lastly, the third mechanism proposed that CS can penetrate cell wall of fungi and bind to DNA. This will inhibit the synthesis of mRNA and, thus, affect the production of essential proteins and enzymes [17,18].

Nanoparticles (NPs) have the unique chemical and physical properties. The size reduction induces to increase surface area to volume ratio as well as increase contact surface with microbes and permeate into cells. Thus, antimicrobial activity increases in comparison with raw materials. Chitosan nanoparticles (CS/NPs) have been recently reported as antimicrobial agents. Although, information is limited on the antifungal activity of CS/NPs against phytopathogenic fungi. Inhibition effect of CS/NPs on the growth of fungi, M. phaseolina, Aspergillus niger and Pyricularia grisea, Alternaria solani, F. oxysporum was observed by Saharan et al. [19], Ing et al. [20] and Sathyabama and Parthasarathy [21], respectively.

In the present study, the effect of CS and CS/NPs investigate on the radial mycelial growth, number of colonies formed and percentage of spore germination of F. graminearum under different concentrations in vitro. In greenhouse, effect of these treatments investigate on the disease severity percentage of FHB and mycelial growth of F. graminearum in the plant cells.

2. Materials and methods

2.1. Materials

CS (Low molecular weight, 161 kDa, 90% N-deacetylation) was purchased from Biobasic (Canada). Fungus agent of FHB disease (Fusarium graminearum) was provided by the Seed and Plant Improvement Institute, Karaj, Iran and was subcultured on Potato Sucrose Agar (PSA).

2.2. Preparation of CS solution

CS was dissolved in 0.5% v/v acetic acid (Merck, Germany) aqueous solution at concentration 0.5%w/v. CS solution was diluted to different concentrations of 5000, 2000, 1400, 1200, 1000, 900, 700, 500, 300 and 100 parts per million (ppm) using distilled water at room temperature (RT) (25 °C). 0.5% v/v acetic acid aqueous solution and tilt fungicide (based on used dose in field, 0.5 ml/100 ml) were used as control and positive control, respectively. The pH of CS solution and acetic acid aqueous solution was about 5.

2.3. Preparation of CS/NPs

CS/NPs were prepared according to the ionic gelation of CS with sodium tripolyphosphate (STPP) (Pyreongtak, Korea) anions. CS was dissolved in 0.5% v/v acetic acid aqueous solution at concentration 0.5%w/v. Aqueous solution of STPP 1 ml with 0.5%w/v concentration was added through a syringe needle into 3 ml CS solution under stirring at RT. The phenomena observed was in aqueous solution. NPs were formed spontaneously upon incorporation of 1 ml STPP solution into 3 ml CS solution. The resultant NPs were then collected by centrifugation model L5-65 (Beckman, USA) at 25000 rpm for 30 min. The supernatants were discarded, and the NPs were redispersed in distilled water. The colloidal CS/NPs solution was diluted to different concentrations of 5000, 2000, 1400, 1200, 1000, 900, 700, 500, 300 and 100 ppm using distilled water at RT. Distilled water and tilt fungicide were used as control and positive control, respectively. The pH of CS/NPs solution and distilled water was about 6.5.

2.4. Characterization of NPs

2.4.1. Dynamic light scattering (DLS) measurements

DLS was used for the measurement of particle size, polydispersity index (PDI) and zeta potential of NPs by Zetasizer model PSS0012-22 (Malvern, UK). The analysis was performed at a scattering angle of 90° at RT. Sample was appropriately diluted with distilled water prior to measurement.

2.4.2. Field emission scanning electron microscopy (FE-SEM) observation

FE-SEM was used to study the surface morphology of NPs. A drop of sample was poured on aluminium stubs and dried at RT for overnight. Then, it was coated with gold using a sputter coater model DSR1 (Nano-Structured Coatings Co., Iran). The sample was examined using FE-SEM model S-4160 (Hitachi, Japan) with 25 kV accelerating voltage.

2.5. In vitro assay

2.5.1. Inhibition rate percentage of radial mycelial growth

Two milliliter of different concentrations of CS and CS/NPs were poured into the Potato Dextrose Agar (PDA) media after plating into each Petri dish. The media containing these treatments were incubated at RT. After 24 h of incubation, an agar plug of 5 mm diameter containing fungus was inoculated simultaneously at the center of each Petri dish and incubated at 25 °C. After 5 days of incubation, inhibition zones were measured. The test was repeated twice and each treatment replicated three times. The percent inhibition rate was calculated by using this formula [22]. % Inhibition rate = radial mycelial growth in control plate–radial mycelial growth in CS and CS/NPs treated plates/radial mycelial growth in control plate × 100.

2.5.2. Preparation of inoculum of F. graminearum

2.5 gm straw debris of wheat and 120 ml distilled water were added to 250 ml flasks and autoclaved for 2 days, successively. A fungus plugs from an actively growing culture were aseptically placed in flasks. Flasks were shaken vigorously on shaker for 4–7 days at 25 °C and then spore suspensions filtered. Conidia were counted by haemocytometer (Zeiss, Germany). A number of conidia were 5 × 10⁴ conidium per ml.

2.5.3. Inhibition rate percentage of number of colonies formed

500 μl of the conidial suspension was mixed with different concentrations of CS and CS/NPs to a final volume of 2 ml. Conidial suspension was also prepared with distilled water and 0.5% v/v acetic acid aqueous solution as controls of CS/NPs and CS, respectively. Also, tilt fungicide was used as positive control. All treatments were incubated at 25 °C for 24 h. Aliquots of 50 μl of each dilution was spread on PDA and incubated at 25 °C. The number of colonies formed on plates was counted after 5 days. The test was repeated twice and each treatment replicated three times. The percent inhibition rate was calculated according to this formula. % Inhibition rate = the number of colonies formed in control plate–the
number of colonies formed in CS and CS/NPs treated plates/the number of colonies formed in control plate \( \times 100 \).

2.5.4. Inhibition rate percentage of conidial germination

500 \( \mu \)l of the conidial suspension was mixed with different concentrations of CS and CS/NPs (100, 500, 1000 and 5000 ppm) to a final volume of 2 ml. Conidial suspension was also prepared with distilled water and 0.5\% v/v acetic acid aqueous solution as controls of CS/NPs and CS, respectively. Also, tilt fungicide was used as positive control. All treatments were incubated at 25\% C for 4 and 24 h. They were stained in 1\% safranin (Merck, Germany) and 1\% methyl blue (Merck, Germany) for 10 and 2 min, respectively. 20 \( \mu \)l of each dilution was poured on the slides and the number of germinated conidia were determined at 400\( \times \) and 1000\( \times \) magnification under Olympus bright field microscopy model BX51 (Olympus, Germany). The percent inhibition rate of spore germination over control was calculated according to this formula [19],

\[
\text{Inhibition rate} = \frac{\text{the number of germinated conidia in control} - \text{the number of germinated conidia in treatments of CS and CS/NPs}}{\text{the number of germinated conidia in control}} \times 100
\]

2.5.5. Samples processing of mycelia for studies by light microscopy

Two ml solution of different concentrations (100, 500, 1000 and 5000 ppm) CS and CS/NPs were applied over fully-developed F. graminearum mycelia grown on PDA using a sprayer. The application was done with the time interval of 1, 3 and 5 days and fungal mycelium treated with 0.5\% v/v acetic acid aqueous solution and distilled water were cultivated as controls. Each treatment was incubated at RT. The entire specimens were stained in 1\% safranin and 1\% methyl blue for 15 and 3 min, respectively. The specimens were observed by light microscopy model BX51 (Olympus, Germany).

2.6. Greenhouse trials

To determine the efficacy of CS and CS/NPs against FHB disease in the greenhouse, an experiment was carried out in greenhouse (Seed and Plant Improvement Institute, Karaj, Iran) in 2015. Inoculation was done at anthesis by injection of 10ul of suspension at concentration of 5 \( \times \)10\(^4\) conidium per ml in medial spikelets using point inoculation method. CS and CS/NPs were used at 1000 and 500 ppm (based on MIc\(_50\), or the minimum concentration of the treatment that is needed to inhibit 50\% of the mycelial growth of fungus, and optional concentration in test of inhibition of radial mycelial growth of F. graminearum) simultaneously. The treatments were applied on the spikelets of Falat variety (susceptible variety) 0, 3 and 5 days after inoculum inoculation by spraying. Each treatment comprised of 18 spikes. Distilled water and 0.5\% v/v acetic acid aqueous solution were used as negative controls for CS/NPs and CS, respectively. Fungicide ‘Tilt’ was used as positive control. The number of infection spikelets were counted 2, 3 and 4 weeks after fungus inoculation.

2.7. Processing of leaf or infected spikelet tissue samples for detection NPs or fungus

The spikelet or leaf fragments (approximately 5–7 mm\(^2\)) with visual symptoms of FHB on spikelets were collected at 4, 8, 24, 48, 72, 84 and 120 h post inoculation (hpi). The fragments were placed in plastic vials containing 20 ml glutaraldehyde (Merck, Germany) 4\% with pH 7–7.4 and stored at 4 °C for 24 h or one week in refrigerator (Electro Steel, Iran).

2.7.1. Processing of spikelet or leaf samples for studies by light microscopy

The entire specimens were rinsed two times by distilled water and subsequently fixed in 1\% osmium tetraoxide (Sigma Aldrich, Germany) for 1 h. After washing with distilled water, the specimens were dehydrated in a graded ethanol (Merck, Germany) series up to 100\%, and embedded in graded xylene (Merck, Germany)/paraffin (Merck, Germany) series. Blocks containing spikelet or leaf fragments were cut to longitude or cross sections that were 7–10 \( \mu \)m by rotary microtome model RM2235 (Leica, Germany). Then, they were stained with 1\% safranin and 1\% methyl blue for 15 and 3 min, respectively. The specimens were observed by light microscopy model BX51 (Olympus, Germany).

2.7.2. Processing of spikelet or leaf samples for studies by transmission electron microscopy (TEM)

For TEM observations, the leaf or spikelet fragments were rinsed two times by 0.1 M cacodylate buffers (Sigma Aldrich, Germany) and subsequently fixed with 1\% osmium tetraoxide for 1 h at 20 °C. Afterwards, they were rinsed by 0.1 M cacodylate buffer, dehydrated in a graded ethanol series for 10 min and embedded in Epon resin 812 (Shell Chemicals, USA). Ultra-thin sections (70–120 nm thick) were cut with a diamond knife model Omu3 (Reichert, Austria) and were collected in Formvar coated nickel grids (Sigma Aldrich, Germany). They were contrasted in uranyl-lacate (Sigma Aldrich, Germany) and lead citrate (Sigma Aldrich, Germany), washed and then examined in a TEM model EM-900 (Zeiss, Germany) at 60 kV.

2.8. Counting the number of infected parts of spikelets

The number of infected parts of spikelets treated with CS and CS/NPs and non-treated with these treatments were counted by visual symptoms and light microscopy, after 4, 8, 24, 48, 72, 84 and 120 h post inoculation. The observation of infected spikelet parts by light microscopy was previously explained.

2.9. Data analysis

All experiments were arranged in factorial in the basis of completely randomized block with three replications per treatment. Calculations were done using the software MSTAT-C (Michigan University, USA). Results were expressed as the mean values ± standard deviation and compared by Duncan’s multiple range test (DMRT) at \( p = 0.01 \).

3. Results

3.1. Size and zeta potential of CS/NPs

The preparation of CS/NPs is based on an ionic gelation interaction between positively charged CS and negatively charged STPP at RT [23]. The mean size and size distribution of each batch of NPs suspension was analyzed using the zetasizer analysis. The size distribution profile, as shown in Fig. 1, represents a typical batch of CS/NPs with a mean diameter of 180.9 nm. Narrow size distribution (polydispersity index) these NPs was 0.31 (Fig. 1). Zeta potential, that is, surface charge, can greatly influence particle stability in suspension through the electrostatic repulsion between particles. It can also determine NPs interaction in vivo with the cell membrane of fungi, which is usually negatively charged. Fig. 1 shows that the surfaces of CS/NPs have a positive charge about 45.6 mV.
3.2. Structure analysis by FE-SEM

FE-SEM image of CS/NPs shows spherical shaped nano structures (Fig. 2). Specimens were observed on a FE-SEM model S-4160 (Hitachi, Japan) at an accelerating voltage of 25 kV.

3.3. Inhibition of radial mycelial growth of F. graminearum on PDA medium by CS and CS/NPs

CS and CS/NPs were used to inhibition of mycelial growth of F. graminearum. Distilled water and 0.5% v/v acetic acid aqueous solution were used as controls of CS/NPs and CS, respectively. These treatments showed various levels of inhibition on mycelial growth of F. graminearum (Table 1 and Fig. 3). Significant inhibition in mycelial growth of F. graminearum supplied with 500–5000 ppm CS/NPs on PDA was evident. The most inhibition was observed more than 85% inhibition on PDA treated with 5000 ppm CS/NPs. The minimum and maximum inhibitory concentration of CS/NPs were 100 and 5000 ppm with percentage of reduction growth %26.6 and %85.1, respectively. Whereas, tilt fungicide reduced %72.55 of fungus growth in concentration of 0.5cc/100cc (based on used dose in field). Also, MIC50 was used as a measurement for the antifungal activity of each treatment. MIC50 of CS/NPs was 500–5000 ppm.

However, CS with all concentrations inhibited mycelial growth of F. graminearum but the results were not as significant as CS/NPs. The antifungal activity of CS did not increase with increasing CS concentrations ranging between 1200 and 5000 ppm. The highest inhibition observed on PDA was on 1000 ppm concentration of CS (68.18%) and lowest inhibition was observed with 100 ppm (17.07%). Therefore, the results showed the inhibition rate varies with the selection of treatment and concentration of them. MIC50 of CS was 1000 ppm. Any sample that has a smaller MIC value is considered to exhibit a stronger antifungal effect. In this experiment, CS/NPs was the most favorable treatment for the suppression of F. graminearum in vitro. It was an effective antifungal agent with MIC as low as 500 ppm. CS required a higher concentration to inhibit 50% of growth of fungus. Based on MIC50 and optional concentration 1000, 500 and 500, 1000 ppm concentrations of CS and CS/NPs were selected for greenhouse trials, respectively.

3.4. Inhibition of the number of colonies formed of F. graminearum on PDA medium by CS and CS/NPs

The number of colonies were counted 5 days after incubation on CS and CS/NPs supplemented medium. CS and CS/NPs showed various levels of inhibition of the number of colonies of F. graminearum (Table 1 and Fig. 3). The number of colonies was decreased with all concentrations in comparison with control in treatment of CS/NPs. The most inhibition was observed 43.95% inhibition on PDA treated with 5000 ppm CS/NPs. The minimum and maximum inhibitory concentration of CS/NPs were 100 and 5000 ppm with percentage of reduction the number of colonies %51.1 and 43.95%, respectively. Whereas, tilt fungicide reduced %100 of the number of colonies in concentration of 0.5cc/100cc.

CS did not inhibit the number of colonies formed of F. graminearum in all concentrations. CS concentrations ranging between 100 and 300 ppm could not inhibit of colony formation in comparison with the control sample. In addition to, these concentrations induced increasing of the number of colonies. The maximum inhibitory concentration of CS was 5000 ppm with percentage of reduction the number of colonies %72.8. The growth inhibition with CS and CS/NPs were dependent on concentration. Moreover, the results showed the inhibition rate varies with the selection of treatment.

3.5. Inhibition of conidial germination of F. graminearum by CS and CS/NPs

In this study, the inhibition effect of CS and CS/NPs on conidial germination of F. graminearum was determined and shown in Fig. 4 in comparison with 0.5% v/v acetic acid aqueous solution and distilled water, respectively. CS markedly reduced conidial germination of F. graminearum than NPs, in both 4 and 24 h incubation. CS with 100, 500, 1000 and 5000 ppm concentrations inhibited spore germination by 12, 17, 23, 28% and 30, 42, 65, 75% in 4 and 24 h incubation, respectively. The maximum of inhibition percentage
Fig. 3. Effect of CS and CS/NPs on radial mycelial growth and number of colony formation of *F. graminearum* after 5 days. Number of colony formation on PDA medium. Suspension of fungal spores soaked for 24 h at different concentration of CS and CS/NPs. 50ul of treated spores spread on PDA medium. A = 500 and B = 1000 ppm concentrations of CS/NPs, C = control (water), D = 500 and E = 1000 ppm concentrations of CS, F = control (0.5% v/v acetic acid aqueous solution). Radial mycelial growth on PDA medium containing concentrations of CS and CS/NPs. G = 500 and H = 1000 ppm concentrations of CS/NPs, I = control (water), J = 500 and K = 1000 ppm concentrations of CS, L = control (0.5% v/v acetic acid aqueous solution).

of conidial germination was obtained in 5000 ppm (75%) concentration, in 24 h incubation (Fig. 4). In addition to, CS was caused deformation and dehydration of spores.

CS/NPs inhibited of conidial germination in all concentrations but 100 ppm in 4 h incubation. The maximum of inhibition percentage of conidial germination was in 5000 ppm (45%) concentration, in 24 h incubation (Fig. 4). The results showed that CS treatment suppressed germination and was found to be more effective than NPs. Also, the incubation time was effective in conidial germination. In both treatments, inhibition percentage of conidial germination in 24 h was more than 4 h. Complete spore germination was observed at distilled water.

3.6. Effect of CS and CS/NPs on mycelial morphology of *F. graminearum*

In previous studies, we showed that CS/NPs penetrated into fungal cells and effected on vacuoles and other organelles. They disrupted cell membranes and induced leakage cytoplasm. In this study, the light microscopic observation revealed that CS and CS/NPs clearly damaged the hyphae, while hyphae treated with water (control) appeared to remain intact. In comparison with CS, 0.5% v/v acetic acid aqueous solution (control) damaged the hyphae very few (Fig. 5). The damage on the hyphae increased by increment of time and concentration of CS and CS/NPs. The positive charge of nanoparticles enable them to interact with negatively charged phospholipid components of fungus membrane. This increased per-
Fig. 4. Effect of CS and CS/NPs on conidial germination of *F. graminearum* after 4 h (A) and 24 h (B) of incubation. Bar shows mean ± standard deviation.

Fig. 5. Light microscopy of hyphae of *F. graminearum* treated with CS and CS/NPs. Fungal hyphae grown on potato dextrose agar plates were sprayed with either water (A) and 0.5% v/v acetic acid aqueous solution (D) as controls of CS/NPs and CS, respectively. Treated mycelium with CS/NPs one (B), three days after incubation (C) and treated mycelium with CS three (E) and five days after incubation (F) in concentration of 1000 ppm.

Meability of membrane and caused the leakage of cellular contents and also, some NPs penetrated into hyphae (Fig. 5B). Fungal hyphae was sunken after 3 days (Fig. 5C). The fungal hyphae observed 3 days after the treatment of CS showed deformities in mycelial growth and dehydration (Fig. 5E). Some hyphae were collapsed at 5 days (Fig. 5F). Therefore, in addition to concentration, treatment time plays an important role in inhibition of pathogen.
3.7. Effect of CS and CS/NPs on FHB disease in the greenhouse

The inhibition effect of CS and CS/NPs was analyzed against FHB disease on wheat in greenhouse of Seed and Plant Improvement Institute, Karaj, Iran, in 2015 (Figs. 6 and 7). In comparison to controls, all treatments suppressed pathogen attack on plants. Table 1 fungicide in concentration of 0.5cc/100cc (based on used dose in field) showed the lowest percent of disease severity in plants, 0.65%, 0.33%, and 2.19%, in 2, 3 and 4 weeks after fungus inoculation, respectively. Also, CS/NPs had low percent of disease severity as well as CS, in 2 and 3 weeks after fungus inoculation. Disease severity percentage in this treatment and CS was 19.47%, 26.87% and 22.01%, 28.74%, in 2 and 3 weeks after fungus inoculation, respectively. Whereas, 4 weeks after fungus inoculation, disease severity percentage by CS/NPs and CS was 41.77% and 45.91%, respectively. The maximum of disease severity percentage was obtained in 4 weeks after fungus inoculation, in control plants treated with distilled water (94.25%) and 0.5% v/v acetic acid aqueous solution (87.34%). Also, disease severity percentage increased in course of time and 4 weeks after fungus inoculation (Fig. 6A and B). The results indicated that CS and CS/NPs could control FHB disease in comparison to controls.

The results showed that disease severity percentage reduced when CS and CS/NPs were applied before the fungus inoculation on the plants (Fig. 6C and D). Plants treated with CS/NPs before and 3 to 5 days after fungus inoculation showed 29.37% and 28.56% to 30.19% disease severity, respectively. Whereas, plants treated with CS after fungus inoculation showed the more percent of disease severity than CS/NPs. Plants treated with CS before and 3 to 5 days after fungus inoculation showed 25.46% and 32.11 to 39.08% disease severity, respectively (Fig. 6C and D). In both treatments, plants treated with 500 ppm had the more percent of disease severity than 1000 ppm (Fig. 6E).

3.8. Detection of CS/NPs in plant tissue and cells by light microscopy and TEM

No particles were detected on control sections of leaf and spikelet (Fig. 8A, E, G). 24 h after the spraying, aggregates of dense material were detected in cells between and inside vascular tissues of leaf and spikelet, on a light microscopy under phase contrast (Fig. 8B and F). Analysis of same specimens by TEM unambiguously revealed numerous NPs close together (Fig. 8C and H), these would correspond to the aggregates observed on the light microscopy. A high magnification of the linear-marked regions in Fig. 8B and F shows that individual round-shaped NPs can be clearly resolved (Fig. 8C, D, H). The diameter of the NPs detected on the TEM was measured. The data were grouped in consecutive intervals spanning 10 nm and the relative frequencies were represented in a histogram (Fig. 9). The distribution of sizes corresponded to sections of the NPs at different levels with maximum values at 100 nm and the most percent of size of NPs was 70 nm. However, the most percent of these NPs in solution was 250 nm.

3.9. Detection of pathogen growth in plant tissue and cells by light microscopy and TEM

At 8 hpi, F. graminearum conidia germinated inside or around the anther (Fig. 10A). At 24 hpi, masses of hyphae were discovered growing extensively in the whole spikelet. The hyphae successively advanced to the palea and lemma parts (Fig. 10B). From 24–48 hpi, numerous growing mycelia completely occupied the anther (Fig. 10C). Then the hyphae were starting cellular penetration, it vigorously invaded parenchyma cells, especially on the palea (Fig. 10D). At 72 hpi, masses of mycelium further spread in the outer space of the spikelet, the glume was gradually colonized...
by pathogen (Fig. 10E). Whereafter the entire spikelet was colonized by *F. graminearum*. From 72–84 hpi, masses of mycelium were spread in the rachis. At 120 hpi, masses of mycelium were spread in the first and second floret (Fig. 10F). Also, the results indicated that mycelium transited to other cells through cell wall or plasmodesmata and all types of cells infected.

Expansion of *F. graminearum* in the spikelets that CS and CS/NPs were applied before or a few moment after fungus inoculation on the them exhibited a few difference from spikelets without these treatments. First, at 8 hpi, somewhat more germinated spores were observed in the spikelets not treated with CS and CS/NPs than spikelets treated with these treatments. Second, hyphal colonization of the spikelets not treated with CS and CS/NPs was somewhat faster than spikelets treated with these treatments. The outer space of the spikelet (glume) and rachis were infected after 72 and 72 to 84 hpi, respectively. Wherease, glume and rachis of spikelets treated with CS and CS/NPs were infected after 84 hpi. At 48 hpi, three parts of spikelets treated with CS and CS/NPs were infected

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**Fig. 6.** Effect of different treatments on disease severity of FHB in different times of disease record (A and B), different times of application (C and D) and different concentrations (E). Control (W): Distilled water, Control (W + A): 0.5% v/v acetic acid aqueous solution. Different letters show significant levels at *p* = 0.01. Vertical bars represent the standard deviation.
as well as spikelets non-treated with these treatments. After 72 to 120 hpi, the number of infected parts of spikelets non-treated with these treatments increased faster than spikelets treated with these treatments (Fig. 11). It is concluded that the expansion of *F. graminearum* in the spikelet was effectively influenced by usage of CS and CS/NPs.

At 24 hpi, fungal hyphae penetrated into the cells through stoma (Fig. 12A). At 48 hpi, fungal hyphae grew intra- and intercellularly in the spikelet tissue however, the distribution of the fungal hyphae in the spikelet was not uniform. Fungal hyphae extensively colonized the cells both vertical and horizontally and produced conidium in the cells, after 72 hpi (Fig. 12B). At 120 hpi, fungal hyphae developed into cells and chloroplasts started to dehydrate and deformate (Fig. 12C). Also, CS/NPs effect on hyphal growth was studied into plant cells. CS/NPs caused hyphal deformation and dehydration and
prevented hyphal growth and some hyphae were collapsed after 72 and 120 hpi (Fig. 12D and E).

4. Discussion

The antifungal activity of CS/NPs against *F. graminearum* in both *in vitro* and in greenhouse experiments was evaluated. Results obtained in this study confirm that CS/NPs have significant inhibitory effects on the fungal growth, colony formation and conidial germination of *F. graminearum*. According to results, the antifungus activity of CS/NPs are significantly higher than CS in inhibition test of radial mycelial growth of *F. graminearum*. NPs induce the changes in morphology, structure and physiology of free form of materials [24]. CS/NPs exhibit higher antifungus activity than CS on account of the special character of the NPs [25]. The negatively charged surface of the fungal cell is the target site of the polycation [26]. Therefore, the polycationic CS/NPs with higher surface charge density interact with the fungus to a greater degree than CS itself. Because of the larger surface area of the CS/NPs, NPs could be tightly adsorbed onto the surface of the fungal cells so as to disrupt the membrane, which would lead to the leakage of cellular contents, thus killing the fungal cells [14,15,25]. CS/NPs penetrate into fungal cells and tightly bind nucleic acids via electrostatic interaction between cationic NPs and anionic DNA [27,28]. This suggest that they may cause a variety of damages and selective inhibitions such as inactivation the synthesis of essential mRNA encoded by various genes required for important metabolic and infectious processes of the microorganism.
In inhibition test of radial mycelial growth of *F. graminearum* was determined that the antifungal activity of CS is not related to its concentration, and may be it attributed to the increased viscosity of the CS solution at higher concentration, which restrict the number of effective contacts of CS with *F. graminearum* cell surface [29]. CS significantly reduced the number of colonies of fungus and percentage spore germination. A number of studies have shown that CS, at defined concentrations, presents antimicrobial properties [30–33].

Effect of CS/NPs on the hyphal growth is more than spores in contrast with CS itself. NPs have high surface area to volume ratio and their contact with fungal cells increases and permeate into cells. On the other hand, the hyphae have high surface area to spores and more NPs adhere to it and significantly permeate into hyphal cells. Palma-Guerrero et al. [34] also reported that spores of the fungal pathogens, *F. oxysporum* and *Verticillium dahliae* were clearly more sensitive to CS than hyphae.

Structural studies showed that treatment with CS and CS/NPs induce a series of morphological and structural modification, leading to disorganized hyphae associated with inhibition of fungal growth. This was linked to the polycationic properties of CS and CS/NPs, allowing for changes in term of membrane permeability and leakage of cellular contents [35].

Against fungi, it seems that CS is likely to operate indirectly via other means such as the enhancement of host resistance [36]. CS, when applied to plant tissues, often agglutinate around the penetration sites and isolate the penetration site through the formation of a physical barrier preventing the pathogen from spreading and invading other healthy tissues. Usually, around the isolated zones, hypersensitive reaction occurs and accumulation of H$_2$O$_2$ is associated with plant defense response to pathogen invasion. This biopolymer stimulates the production of reactive oxygen species (ROS) such as H$_2$O$_2$ and the accumulation of pathogenesis related (PR) proteins such as chitinase, induces the formation and accumulation of phenolic compounds such as phytoalexins in various host cells, promotes lignification, inhibits the action of proteinase and activates peroxidase, superoxide dismutase and catalase enzymes [37–40].

Plants treated with CS and CS/NPs before fungus inoculation had the low percent of disease severity. Application of these treatments before fungus inoculation activate the plant defence systems such as induce resistance [36] and prevent of pathogen development. In addition to, after fungus inoculation, the fungus sufficiently grow in plants and application of these treatments specially CS does not efficiently effect on the hyphal growth.

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**Fig. 11.** The number of infected parts of spikelets treated with CS and CS/NPs and non-treated with these treatments in different hours post inoculation. 1: Anther, palea, lemma, 2: Anther, palea, lemma, glume, 3: Anther, palea, lemma, glume, rachis; 4: Anther, palea, lemma, glume, rachis, second floret; 5: Anther, palea, lemma, glume, rachis, first and second floret. Bar shows mean ± standard deviation.

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**Fig. 12.** TEM and Light microscopy micrographs of *F. graminearum* mycelial growth and penetration into cells and effect of CS/NPs on mycelial growth. A. Penetration of fungus of stoma observed at 24 hpi. B. Production of conidium inside spikelet cells, 72 hpi. C. Deformation and dehydration of chloroplasts under effect mycelium development 120 hpi. D and E. collapse of hyphae by CS/NPs 72 and 120 hpi, respectively. (Scale bars of A = 10 μm, B, C, E = 4 μm and D = 2 μm). H = Hyphae, S = Stoma, VA = Vacuole, C = Conidium, M = Mitochondria, P = Chloroplast.
CS/NPs have been widely investigated as nanocarrier in animal cells [41]. But there is not any report about penetration and transportation of CS/NPs inside whole plant. In this work, semi thin and ultra thin sections from blocks with same specimen embedded in paraffin and resin with different staining ways were used to detect NPs in plant tissue and cells by light microscopy and TEM, respectively. The cell walls and waxes act as physical barrier and prevent of penetration of NPs into plant cells thus particles bigger than 100 nm can not penetrate into plant cells.

Investigation of the pathogen development in the ear can help to understand disease management. The spikelet is an important part of the ear and determines the grain production. In addition to, the spikelet is considered as the entry point for pathogens. The flowering stage is favourable for pathogen invasion, spores of *F. graminearum* deposit on or in the floral organ for germination and then initiate infection during the anthesis period [42,43]. The first infection step on the ear is the anther. In this study, germinated spores were found on the anther at 8 hpi. Also, the anther is the first part of flower which contact with *F. graminearum* inoculum [44]. The hyphae colonize the filament and the stigma, then, penetrate the ovary and colonize the floret bracts including the glume, palea and lemma [45–47]. The present study suggests that anther was covered with conidia after 8 hpi, following the mycelia were found to be thriving around the palea, lemma and glume after 24 and 72 hpi, respectively. Whereafter, spikelets treated with CS and CS/NPs were wholly occupied with *F. graminearum* after 84 hpi. Thus, spikelets treated with CS and CS/NPs were infected slowly.

The rachilla connects spikelet to rachis and stem. It is a key factor to understand the systemic pathogen progress in plant. The vessels inside the rachis are linked to the ones in the rachilla and stem, which forms a path for the fungal expansion in plant. Initially, *F. graminearum* was detected in the vessels of rachis from 72 hpi to 84 hpi, whereas in plants treated with CS and CS/NPs, *F. graminearum* was found in vessels only at 84 hpi. *F. graminearum* clearly spreads fast both intra- and inter-cellularly in the plants without CS and CS/NPs.

5. Conclusion

However, in this study we focused on the inhibition effect of CS and CS/NPs against *F. graminearum* on *PDA in vitro* as well as in greenhouse trials. This study suggests that CS and CS/NPs are effective for the control of FHb disease. Thus, CS and specially CS/NPs prepared by a suitable method can be used as biological pesticide in controlling fungal plant pathogens. The greenhouse study also suggests that, plant protection by CS and CS/NPs is dependent on time period. Plant can be protected of disease with spraying of them at anthesis, twice or thrice in time of plant growing.

In contrast to chemicals, CS has very low toxicity toward mammals. However, with regard to antimicrobial activity of CS and CS/NPs, much experimental trials are needed to understand exact mechanism of penetration and transportation of these treatments into plant cells and their interaction with fungal cells in these cells.

References
