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Research Article

Preparation and Antifungal Activity Investigation of Oligochitosan-Zn^{2+} on *Colletotrichum truncatum*

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This study presents the structural characteristics and the antifungal efficiency of the oligochitosan-Zn^{2+} complexes. Oligochitosan with the average molecular weight of ~5 kDa was effectively prepared by gamma-ray irradiation degradation of chitosan in a solution containing H_2O_2. The oligochitosan-Zn^{2+} complexes with the different molar ratios of -NH_2/Zn^{2+} were prepared by mixing $Zn(NO_3)_2$ into oligochitosan solution. The resultant complexes were characterized by FTIR, XRD, UV-Vis, and ICP-AES. The obtained results demonstrated that Zn^{2+} ions were stably bound with oligochitosan molecules through interacting with -OH and -NH_2 groups. The *in vitro* antifungal effect of oligochitosan-Zn^{2+} complexes was assessed against *Colletotrichum truncatum*, a fungus species causing anthracnose on crops. The antifungal activity was significantly improved as the increase of Zn^{2+} content in the complexes. Particularly, the antifungal efficiency of the complexes reached to 75–100% compared to that of about 12% for oligochitosan. Thus, the addition of Zn^{2+} into oligochitosan strengthens its performance towards antifungal property and bring forward a new approach for progressing biobased materials for controlling plant diseases.

1. Introduction

Colletotrichum truncatum, the primarily causal fungus species of anthracnose, is a severely devastating disease fungus and has a wide host crop range including soybean. According to Gawade et al., the tune of 30–70% of quality as well as quantity of soybean seeds can be lost due to anthracnose disease [1]. However, concerning environment and human health aspects, there are some difficulties to control this pathogen by employing chemical fungicides prevalently adopted in modern agriculture [2]. Therefore, it is necessary to discover other ecofriendly and effective antimicrobial reagents for controlling crop plant diseases.

In recent years, chitosan and its derivatives, the natural polysaccharides, are well known as the efficient and broad-spectrum antimicrobial agents [3, 4]. Moreover, it is also recognized as a marvelous biostimulant on crop plants, and it has the potential in agriculture with regard to controlling disease and promoting growth on plants [5]. The antimicrobial as well as elicitation efficiency of chitosan mainly depends on the concentration and its molecular weight [4–7]. According to the result reported by Dalvi et al., the antifungal efficacy of oligochitosan against *Alternaria solani* was rather strong than that of chitosan [4]. In spite of its beneficial properties, oligochitosan (OC), like other natural oligosaccharides, could be modified for creating new products with the improvement of the desired properties. In biochemistry, zinc constitutes one of the essential elements for living organisms and possesses a strong antimicrobial activity in low concentration. Savi et al. described an efficient

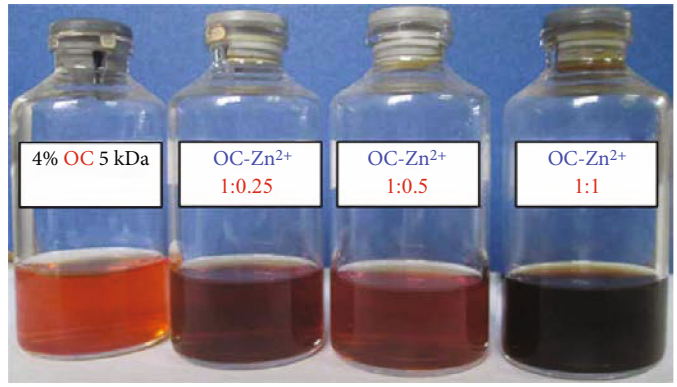

Figure 1: Photographs of OC and OC-Zn^{2+} complexes in solution.

antifungal ability of zinc compounds against *Fusarium graminearum* fungous species at 100 mM concentration [8]. Moreover, Aziz et al. reported that the combination of OC with copper sulfate enhanced the defensive extent and the protective ability of grapevine against the pathogen fungi [9]. Khan et al. also reported an augmentation in antibacterial activity of irradiated chitosan that combined with Zn^{2+} ions [10]. Nevertheless, to the best of our knowledge, the antifungal efficiency of OC-Zn^{2+} complexes on *Colletotrichum truncatum* has not been reported so far.

This work presents the preparation of OC-Zn^{2+} complexes and their structural characteristics. Concurrently, the *in vitro* antifungal efficiency of OC and OC-Zn^{2+} complexes against *Colletotrichum truncatum* (*C. truncatum*) was also investigated.

2. Materials and Methods

2.1. Materials. The OC 4% (*w*/*v*) solution was obtained by the gamma Co-60 ray irradiation degradation method of chitosan solution containing 0.5% (*w*/*v*) H_2O_2 at a dose of 21 kGy [11]. The OC possessed a deacetylation degree of ~88.5% and the weight average molecular weight of ~5.0 kDa. $Zn(NO_3)_2{\cdot}6H_2O$ salt with a purity > 99% and the fungal culture medium of Potato Dextrose Agar (PDA) were purchased from Merck, Germany. The fungal strain of *C. truncatum*, isolated from anthracnose disease in soybean leaves, was kindly provided by the Research and Development Center for High Technology Agriculture, Cu Chi, Ho Chi Minh City, Vietnam.

2.2. Preparation and Characterization of OC-Zn^{2+} Complexes. The OC-Zn^{2+} complexes were prepared by a mixing method [10, 12]. The desired amount of $Zn(NO_3)_2{\cdot}6H_2O$ (corresponding to a molar ratio $-NH_2/Zn^{2+}$ of 1/0.25; 1 : 0.5; and 1/1) dissolved in 100 mL OC solution (equivalent to ~0.02 mole of $-NH_2$). Then, the mixtures were stirred at room temperature for 3 h to obtain OC-Zn^{2+} complex solutions that contained 4% (*w*/*v*) OC and different Zn^{2+} contents as mentioned above. The UV-Vis spectra of OC solution and OC-Zn^{2+} complexes were recorded on an UV-2401PC, Shimadzu, Japan. The OC and OC-Zn^{2+} complexes in powder were also prepared by precipitation with ethanol (1 V sample+8 V absolute ethanol), centrifugation, drying, and grinding into fine powder for measuring FTIR, XRD, and ICP-AES. The FTIR spectra of OC and OC-Zn^{2+} complexes in KBr pellet were measured in the range of 4000–400 cm^{-1} on a FTIR spectrophotometer (FTIR 8400S, Shimadzu, Japan). The content of zinc in OC-Zn^{2+} was determined by inductively coupled plasma-atomic emission spectroscopy (ICP-AES) on a Perkin-Elmer, Optima 5300 DV. X-ray diffraction (XRD) of samples was obtained on a D8 Advance Bruker, Germany.

2.3. The In Vitro Antifungal Activity Assay. The antifungal activity of OC and OC-Zn^{2+} complexes on the mycelial growth of *C. truncatum* was performed by a culture medium toxicity method [13] as follows: the solutions of OC and/or OC-Zn^{2+} complexes were added into PDA medium solution to reach a final OC concentration of 250, 500, and 1000 mg OC per litter. Then, the prepared mixtures and the medium PDA solution without studied reagents were autoclaved for 15 min at 121°C for sterilization. Afterward, 15 mL of the sterilized mixture solutions was poured into petri dishes with 90 mm in diameter and kept to be solidified. Subsequently, the mycelial discs of 6 mm in diameter of *C. truncatum* grown on PDA plates were served from the margins of the colony and placed in the center of each PDA plate containing different concentrations of OC and/or OC-Zn^{2+}. The plates were inoculated at 30°C for 5–10 days until the mycelium of *C. truncatum* reached the edges of the control plate (without reagent addition). The diameter of the mycelial radial zones on plates was measured and antifungal efficiency (AE) was calculated as follows [14]:

$$\mathrm{AE}\,(\%) = 100 \times \frac{D_c - D_s}{D_c}, \quad (1)$$

where D_c and D_s are the diameter of the mycelial radial zone in the control plate and test plate, respectively. The diameter values were expressed as the mean ± SD, calculated from nine plates of each treatment. The mean separation and the significant level were analyzed using the MSTATC software (version 1.2, Michigan, US) with probability values of $p < 0.05$.

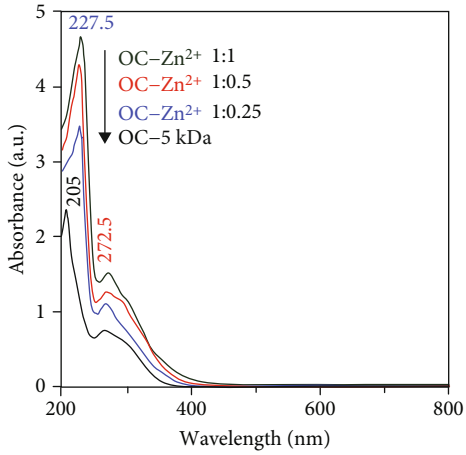

Figure 2: The UV-Vis spectra of OC and OC-Zn^{2+} complexes in solution.

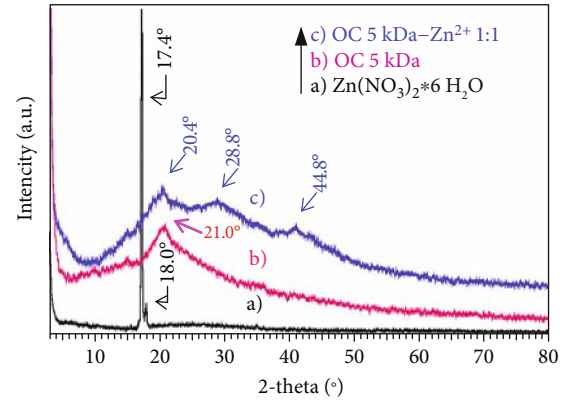

Figure 3: The XRD patterns of OC and OC-Zn^{2+} complex in powder.

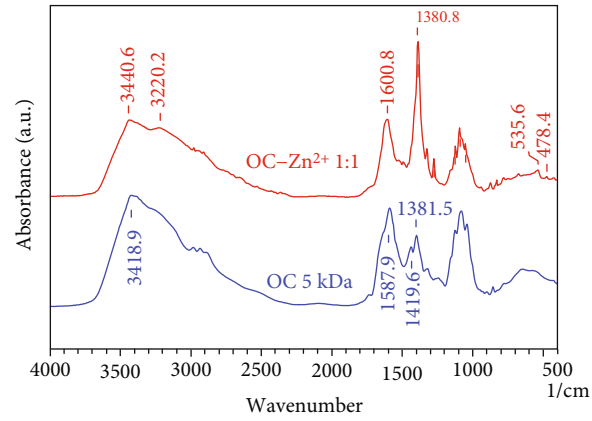

Figure 4: The FTIR spectra of OC and OC-Zn^{2+} complexes in powder.

3. Results and Discussion

3.1. Characteristics of OC-Zn^{2+} Complexes. After adding zinc nitrate, the color of OC solution was turned from brownish-yellow to dark brown and deep brown as an increase of Zn^{2+} concentration (Figure 1).

Furthermore, the results of UV-Vis spectra in Figure 2 showed that the specific peak of N-acetyl-glucosamine and glucosamine residues on OC molecules at around 205 nm [15] was shifted to 227.5 nm in case of OC-Zn^{2+} complex. The peak around 227.5 nm, attributed to the excitation of the OC molecules by the presence of Zn^{2+} ions, was assigned to –O–metal ion bonds as reported by Mekahlia and Bouzid for chitosan-Cu^{2+} complexes [16]. Moreover, the intensity of the peak at ~272 nm that assigned for C=O linkage in carbonyl and/or carboxyl groups [17] was increased with increasing Zn^{2+} content. All changes in color and UV-Vis absorbance of OC-Zn^{2+} complexes can be presumed that the interaction between Zn^{2+} and OC molecules in solution has occurred through coordinate bonds with each other.

The XRD diffraction patterns of $Zn(NO_3)_2{\cdot}6H_2O$, OC, and OC-Zn^{2+} with the molar ratio of 1 : 1 are shown in Figure 3. The $Zn(NO_3){\cdot}6H_2O$ showed two specific sharp peaks at 2θ of 17.4° and 18.0°, which specified for monoclinic crystal type of zinc nitrate salt (COD 9008175 in XRD database). The OC showed a peak at 2θ of 21.0° characterizing for the amorphous structure of OC [17, 18], while the OC-Zn^{2+} complex exhibited three major peaks at 2θ of 20.4°, 28.8°, and 40.8° in XRD pattern. This result can be implied the formation of a new crystalline phase in the complex of OC-Zn^{2+} [10, 12]. To consolidate the zinc ion chelated with OC in complex, the zinc content of complex in powder was analyzed by an ICP-AES technique. The obtained result was of 182 mg Zn·g^{-1} compared with that of 204 mg Zn·g^{-1} of theoretical calculation for the complex sample of OC-Zn^{2+} 1 : 1. This result can be inferred that almost the amount of initial Zn^{2+} (~90%) was able to link to OC in the OC-Zn^{2+} complex.

The FTIR spectra in Figure 4 showed that the main peaks of OC have almost appeared in OC-Zn^{2+} complex. However, in FTIR spectrum of OC-Zn^{2+} complex, the peak at 3419 cm^{-1} assigned for N-H bond and O-H bond stretching vibrations of OC [11, 12] was shifted to longer wavenumber at 3440 cm^{-1}, and concurrently, a new peak at 3220 cm^{-1} also appeared due to OC interacts with Zn^{2+} ion. According to Mekahlia and Bouzid [16], this new peak was a result of the unfolded peak from the O-H band overlapping the stretching band of N-H when OC or chitosan interacts with metal ions. The peak at 1588 cm^{-1} assigned for vibrations of C-O bond in carboxyl and carbonyl on OC molecules [18] was shifted to ~1600 cm^{-1} in the OC-Zn^{2+} sample. Besides, the peak at ~1420 cm^{-1} assigned for plane vibration of the –OH group was disappeared, but the intensity of a peak at 1381 cm^{-1} assigned for $-CH_3$ vibration was increased. In addition, the new peaks at 536 cm^{-1} and 478 cm^{-1}, which were assigned for N-Zn bond and O-Zn bond stretching vibrations [11, 12], were observed in OC-Zn^{2+} complex. According to Wang et al. [12], the complex interaction between Zn^{2+} and chitosan could be described based on the Lewis acid-base theory, in which Zn^{2+} acts as an acid and chitosan as a base. The structure of chitosan-Zn^{2+} belongs to a bridge pattern (Figure 5(a)) and/or a pendant pattern (Figure 5(b)). Those patterns depend on

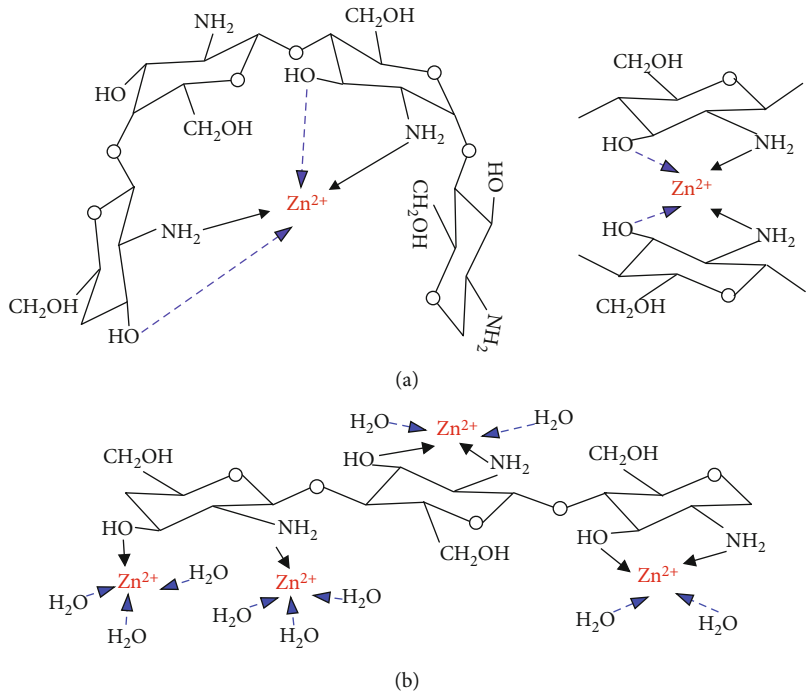

Figure 5: The structure of OC-Zn^{2+} complexes.

Table 1: The antifungal activity on *C. truncatum* of OC, OC-Zn^{2+} complexes, and Zn^{2+}.

Treatment	Contrl.	250 mg·L^{-1}		500 mg·L^{-1}		1000 mg·L^{-1}	
	D (mm)	*D* (mm)	AE (%)	*D* (mm)	AE (%)	*D* (mm)	AE (%)
OC 5 kDa		$75.4^{a} \pm 1.7$	0.3	$74.7^{a} \pm 0.8$	1.2	$66.8^{a} \pm 1.6$	11.6
Zn^{2+} (78.7 mg·L^{-1})	75.6 ± 1.8	—	—	—	—	$44.3^{b} \pm 1.8$	44.1
OC-Zn^{2+} 1 : 0.25		$61.7^{b} \pm 1.9$	18.4	$56.8^{b} \pm 1.7$	24.9	$18.8^{c} \pm 1.5$	75.1
OC-Zn^{2+} 1 : 0.5		$58.7^{b} \pm 1.4$	22.4	$53.7^{c} \pm 1.7$	29.0	$06.3^{d} \pm 0.9$	91.7
OC-Zn^{2+} 1 : 1		$52.3^{c} \pm 2.6$	30.8	$09.1^{d} \pm 1.8$	88.0	<0.5	~100
$LSD_{0.05}$	—	*4.24*	—	*2.47*	—	*1.97*	—

In the same column, values with different letters mean significant difference ($p < 0.05$).

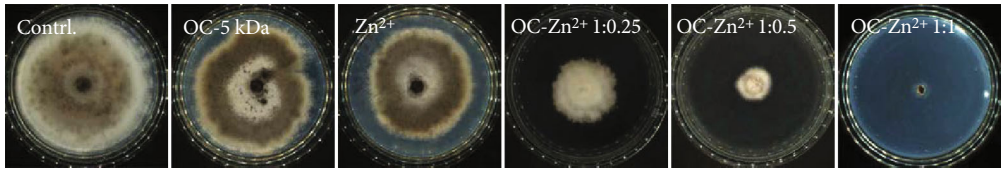

Figure 6: The mycelium growth of *C. truncatum* on PDA plates added the testing substances.

the molar ratio of Zn^{2+} and chitosan. Hence, the low content of Zn^{2+} favors to a bridge chelate pattern.

3.2. Antifungal Activity. The antifungal activity of OC and OC-Zn^{2+} complexes with different concentrations and one test sample of $Zn(NO_3)_2{\cdot}6H_2O$ 358 mg·L^{-1} (equal to the amount of 78.7 mg $Zn^{2+}{\cdot}L^{-1}$ in OC-Zn^{2+} 1 : 0.25 complex tested at 1000 mg OC·L^{-1} concentration) were evaluated against the *C. truncatum* growth on PDA plates. The obtained results in Table 1 and Figure 6 showed that the antifungal activity was enhanced as an increase of OC and OC-Zn^{2+} complex concentration. Also, the complexes of OC-Zn^{2+} with the higher Zn^{2+} content exhibited a stronger antifungal activity. Particularly, the AE values were of 75.1%, 91.7%, and ~100% for OC-Zn^{2+} complexes with a molar ratio of 1 : 0.25, 1 : 0.5, and 1 : 1, respectively. By comparison among tested samples in Table 1, the antifungal activity of OC-Zn^{2+} complex was higher than that of OC and Zn^{2+} alone, particularly the AE values were of 11.6%, 44.1%, and 75.1% corresponding to OC, Zn^{2+}, and OC-Zn^{2+} 1 : 0.25 complex. Interestingly, these results indicated that the AE value of OC-Zn^{2+} complex was higher than the

sum of the AE value of OC and Zn^{2+}. According to Duy et al., a synergistic effect is defined as the simultaneous effect of two factors (or reactants) greater than the sum of the individual effects [18]. Based on the definition and the obtained results, it indicated that the synergistic effect in antifungal efficiency of OC and Zn^{2+} in OC-Zn^{2+} complex against *C. truncatum* has occurred.

The synergistic antifungal activity of chitosan and Cu^{2+} against *Botrytis cinerea* and *Plasmopara viticola* fungi was recognized by Aziz et al. [9]. Wang et al. also reported that the antimicrobial activity of chitosan-Zn^{2+} complexes was higher than that of chitosan (2–8 folds) and Zn^{2+} (4–16 folds) [12]. The reason for the occurrence of the synergistic effect can be explained that by chelating of OC or chitosan with Zn^{2+}, the positive charge on their molecules was strengthened. Therefore, the complexes were more favorable to the interaction with anionic constituents of microbial cells and exhibited high antimicrobial activity [9, 10, 12].

Moreover, it is worth to note that the antimicrobial activity of chitosan depends on the degree of deacetylation, molecular weight, microbial strain, and medium pH [7, 19]. Furthermore, besides the chitosan from shrimp shells, crab shells, and squid pen, the chitosan derived from different chitin sources particularly *Daphnia longispina* resting eggs [3], scorpions [7], and orthoptera species [20] also manifested significant antimicrobial activity that can be suitably used to prepare the complexes with Zn^{2+} for applications as effective bactericides.

4. Conclusions

A series of OC-Zn^{2+} complexes with different molar ratios was successfully prepared. The structural properties of complexes, in which Zn^{2+} ions interacted with the OC molecules through coordinate bonds with hydroxyl, amino, carboxyl, and carbonyl groups, were characterized by UV-Vis, FTIR, XRD, and ICP-AES techniques. The prepared OC-Zn^{2+} complex exhibited a synergistic effect in antifungal activity against *C. truncatum*. Therefore, OC-Zn^{2+} complexes, as an alternative biofungicide, have a potential for application in controlling anthracnose disease caused by *C. truncatum* in crops. However, field experiments on the antifungal effect of OC-Zn^{2+} complexes should be carried out for a practical application in sustainable agricultural production.

Data Availability

The experimental and analytical data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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