PREPARATION OF RADIOLYSIS-DEGRADED OLIGOCHITOSAN, OLIGOCHITOSAN-Zn^{2+} COMPLEX AND THEIR INDUCED EFFECT AGAINST ANTHRACNOSE ON SOYBEAN PLANTS

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Abstract

Background: Anthracnose is a severely devastating disease in soybean cultivation. There are some difficulties to control this pathogen by employing chemical fungicides prevalently adopted in modern agriculture. In recent years, the oligochitosan (OC) and OC-Zn^{2+} complexes have been evaluated as efficient fungicides. However, finding a proper method to produce them is still being actively encouraged.

Objectives: Study on preparation of OC with the low molecular weight and OC-Zn^{2+} complex with the high disease resistance, concurrently, demonstrate the structural characteristics and evaluate the effectiveness of anthracnose resistance on soybean plants.

Methods: The oligochitosan (OC) was prepared by gamma-ray irradiation combined with a small amount of H_2O_2. The OC-Zn^{2+} complex was made by mixing at rT° for 3 h. The prepared samples were characterized by GPC, UV-Vis, XRD, and FT-IR techniques. Eliciting effect against anthracnose was performed in the greenhouse and evaluated by measuring the disease incidence and disease severity of soybean plants sprayed with the OC and/or OC-Zn^{2+} complex.

Results: The prepared OC was almost unchanged in the structural chemical compared to the original chitosan and there was an interaction between Zn^{2+} ions with OC molecules in complex form. Furthermore, the OC-Zn^{2+} complex exhibited a significant improvement in the elicitation effect against anthracnose on soybean plants. Typically, the disease severity was of 35.7% and 13.8% for OC and OC-Zn^{2+}, respectively in comparison of 79.8% for the control.

Conclusion: The OC with Mw ~5000 and the OC-Zn^{2+} complex with the molar ratio of 1/0.5 were successfully produced. The elicitation effect against anthracnose on soybean of OC-Zn^{2+} treating improved significantly. In addition, the complex of OC-Zn^{2+} can be favorably produced on large scale and applied to used in agriculture as a plant elicitor.

Keywords: *Oligochitosan, zinc; anthracnose, gamma-ray irradiation.*

1. INTRODUCTION

The anthracnose on soybean plants due to the causal *Collectotrichum truncatum* fungus is a severely devastating disease in soybean cultivation. According to Gawade et al. (2009) [1], the tune of 30 – 70% of quality as well as quantity of soybean seeds can be lost due to anthracnose disease. However, 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 eco-friendly and effective antimicrobial reagents. In recent years, chitosan and its derivatives are well known as the efficient and broad-spectrum antimicrobial agents. Moreover, it is also recognized as a marvelous bio-stimulant and controlling disease agrochemical on crop plants [3]. The antimicrobial as well as elicitation efficiency of chitosan mainly depends on the concentration and its molecular weight, the lower the molecular weight of chitosan the higher the effect [2-3]. 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 is one of the essential elements for living organisms and possesses a strong antimicrobial activity in low concentration. Savi et al. (2015) [4] described an efficient antifungal ability of zinc compounds against *Fusarium graminearum* fungous species at 100 mM concentration. Moreover, Aziz et al. (2006) [5] reported that the combination of OC with copper sulfate enhanced the defensive extent and the protective ability of grapevine against the pathogen fungi. Khan et al. (2013) [6] also reported an augmentation in antibacterial activity of irradiated chitosan that combined with Zn^{2+} ions. Nevertheless, to the best of our knowledge, the antifungal efficiency of OC-Zn^{2+} complexes on *Collectotrichum truncatum* has not been reported so far.

This work presents the preparation of OC, OC-Zn^{2+} complex and their characteristics. Concurrently, the elicitation effect against anthracnose on soybean of them was investigated.

2. EXPERIMNETAL CONTENT

2.1. Materials and methods: Seeds of soybean variety HL 17-15 were obtained from Hung Loc Agricultural Research Center, Dong Nai, Vietnam. Chitosan in flake form with a degree of deacetylation of ~91.4%; the weight average molecular weight (Mw) of 44.5 $\times 10^3$ g/mol was supplied by a factory in Vung Tau Province, Vietnam. $Zn(NO_3)_2 \bullet 6H_2O$ salt with a purity >99% was purchased from Merck, Germany. The fungal strain of *Collectotrichum truncatum* (*C. truncatum*), isolated from anthracnose disease in soybean leaf, was kindly provided by R&D Center for High Technology Agriculture (AHRD), HCM City, Vietnam.

+ *Preparation and characterization of OC and OC-Zn^{2+} complexes:* A OC 4% (w/v) solution was obtained by gamma Co-60 ray irradiation of chitosan 4% (w/v) solution containing 0.5 % (w/v) H_2O_2 at the dose of 21 kGy [7]. The OC-Zn^{2+} complex was prepared by mixing method [6, 8]. Briefly, 3 g of $Zn(NO_3)_2 \bullet 6H_2O$ (~0.01 mol of Zn^{2+}) was dissolved in 100 ml OC solution (equivalent to ~0.02 mole of –NH_2 groups). Then, the mixture was stirred at room temperature for 3 h to obtain OC-Zn^{2+} complex solution with the molar ratio of 1/0.5 (–NH_2/Zn^{2+}). The UV-Vis spectra of OC and OC-Zn^{2+} complex in solution were recorded on an UV-2401PC, Shimadzu, Japan. The ethanol precipitators of OC and OC-Zn^{2+} complex in powder were characterized using FTIR, XRD [7, 8].

+ *Experimental design and disease resistance assay:* The experiment consisted of three treatments (control – H_2O; OC-100 ppm, and OC-Zn^{2+} with dilution similar to that of OC) was conducted in the greenhouse of the AHRD from Feb. to Apr., 2019 using the drip irrigation technology. Each treatment included three replications (10 plants/rep.) that were arranged as the Completely Randomized Design. Foliar spaying was applied three times after seed sowing of 15, 25 and 35 days. The sprayed volume was of 0.5 L/10 plants that grown in plastic pots (1 plant/pot). 24 h after the third spraying, the plants further were sprayed with a fungal spore suspension of *C. truncatum* (6 $\times 10^4$ spores/mL) with a dose of 0.5 L/10 plants for inoculating. The disease incidence (DS) and disease severity (DS) after 21 days of innoculaing were calculated by using the formulas as presented in the article of Zahid et al. [9]. The values were expressed as the mean ± SD, calculated from three replications of each treatment using the MSTATC software (version 1.2, Michigan, US) with p <0.05.

2.2. Results

+ *Characteristics of OC and OC-Zn^{2+} complex:*

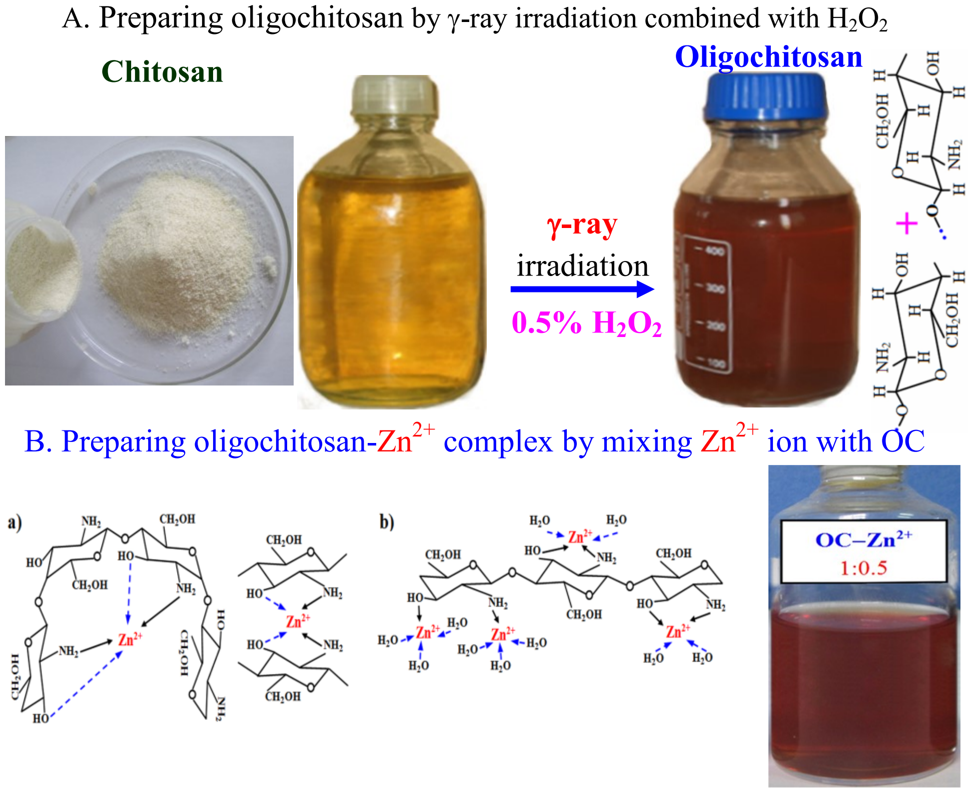

Figure 1. Photographs and molecular structures of chitosan, OC, and OC-Zn^{2+} complex in solution

The color of chitosan solution containing 0.5% H_2O_2 after γ-ray irradiation of 21 kGy was turned from yellow to slightly turbid brown that was being changed to light brown when zinc nitrate was added (Fig. 1). The obtained OC after irradiating possessed a deacetylation degree of ~88.5% and the weight average molecular weight of ~5000, the values were measured by using a GPC technique (Fig. 2) and the FTIR spectrum (Fig. 4) [7, 10].

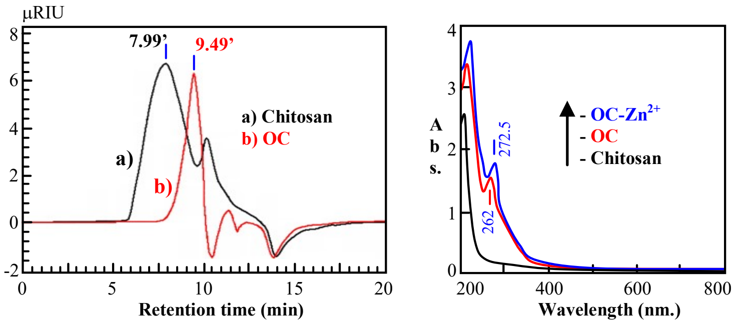

Figure 2. The GPC curves (left) and UV-Vis spectra (right) of OC and OC-Zn^{2+} complexes in solution

The UV-Vis spectra in Fig. 2 showed that the chitosan has no any peak in the range of 200 – 800 nm, while the specific peak of N-acetyl-glucosamine and glucosamine residues on OC molecules at around 205 nm [11, 12] was shifted to 219 nm and 227 nm in OC and OC-Zn^{2+} complex, respectively. Moreover, on the UV-Vis spectra, there are new peaks at 262 nm and 272.5 nm respectively for OC and OC-Zn^{2+}.

The XRD diffraction patterns of $Zn(NO_3)_2$•$6H_2O$, chitosan, OC, and OC-Zn^{2+} samples were shown in Fig. 3. The $Zn(NO_3)$•$6H_2O$ 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). Chitosan has two peaks at 2θ of 10.2° and 20.4°, which are a specific characteristic of semi-crystalline crystical of polysaccharides. The OC showed a peak at 2θ of 21.0° with low intensity, which was typical for amorphous structure of OC [10, 12], while the OC-Zn^{2+} complex exhibited of three major peaks at 2θ of 20.4°, 28.8° and 40.8° in XRD pattern.

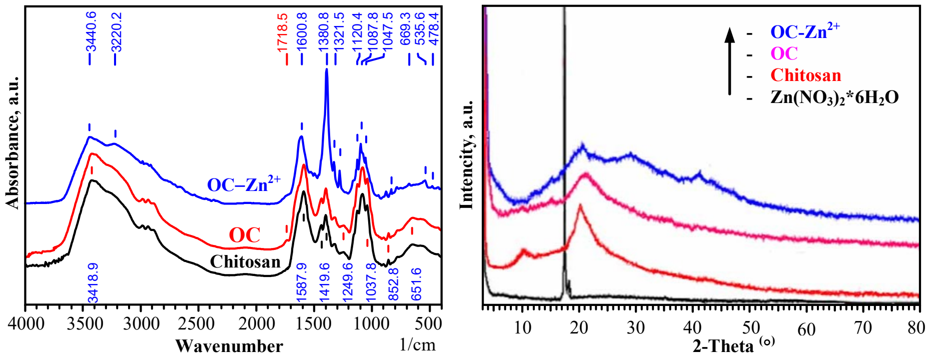

Figure 3. The FTIR spectra (left) and XRD patterns (left) of chitosan, OC, and OC-Zn^{2+} in powder

The FTIR spectra in Fig. 3 showed that the main peaks of chitosan were almost appeared in OC and OC-Zn^{2+} complex. However, there was a new peak at 1718.5 cm^{-1} with the low intensity in case of OC; and two new peaks at 535.6 cm^{-1} and 478.4 cm^{-1} for OC-Zn^{2+}.

+ *Disease anthracnose resistance activity of OC and OC-Zn^{2+}*

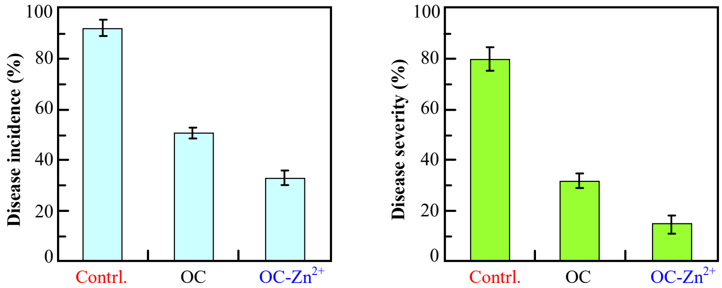

Figure 4. The DI and DS on soybean plants after 21 days inoculating with *C. truncatum*

The obtained results of disease anthracnose resistance on soybean plants artificially inoculated and treated with OC (100 ppm) and OC-Zn^{2+} (the same as the dilution of the OC sample) was presented in Fig. 4. Both DI and DS on soybean treated with OC and OC-Zn^{2+} are decrease, namely, the DI values are 52.2% and 33.3%; the DS values are 35.7% and 13.8% respective to OC and OC-Zn^{2+} in compared to the control (DI = 92%; DS = 79.9%).

2.3. Discussion

The combined γ-ray/H_2O_2 method to produce OC from chitosan in solution is significantly efficient one due to the reduced dose of irradiation and non-toxic by-products from radiolysis of H_2O_2 [10]. In particularity, the prepared OC in this way was almost unchanged in the structural chemical compared to the initial chitosan. Whereas, based on the appearance of new peaks in the UV-Vis and FTIR spectra of the OC at 262 nm and 1718.5 cm^{-1}, which was the specific characteristic peaks of carbonyl/carboxyl groups of terminal monomers, indicated that the much new molecular chains of chitosan was formed. Moreover, the GPC curves and XRD patterns clearly exhibited that molecular weight and crystallinity of the yielded OC were strongly reduced, which means increased solubility. The obtained results are consistent with the previously our researches [7, 10]. For the OC-Zn^{2+}, all the changes on the color; the UV-Vis spectrum (shifting peaks of OC from 219 to 227 nm and 262 to 272.5 nm in OC-Zn^{2+} was attributed to the excitation of the OC molecules by Zn^{2+} [12, 13]); the FTIR spectrum (appeared new peaks at ~536 cm^{-1} and 478 cm^{-1} assigned for N–Zn bond and O–Zn bond stretching vibrations [5, 8]; the XRD pattern (exhibited new peaks on the pattern implied the formation of new crystalline phase in complex of OC-Zn^{2+} [5, 8]) of OC-Zn^{2+} can be suggested that the interaction between Zn^{2+} and OC molecules in solution has occurred through dative bonds with each other. According to Wang et al. (2004) [8], the interaction between Zn^{2+} and chitosan could be described based on Lewis acid-base theory, in which Zn^{2+} acts as an acid and chitosan as a base. The structure of chitosan-Zn^{2+} belong to a bridge pattern (Fig. 1a) and/or a pendant pattern (Fig. 1b). Those patterns depend on molar ratio of Zn^{2+} and chitosan. Hence, the low content of Zn^{2+} favors to bridge chelate pattern. The effects of OC and OC-Zn^{2+} on disease resistance and antifungal are significant improvement. The reasons could be explained that the OC with low molecular weight and good solubility made it easier to interact with microbial cells than that of chitosan. Moreover, an addition of Zn^{2+}, an essential element also possessed a disease resistibility and antifungal for crop plants [4, 6], ameliorated the disease induced-resistance and antifungal of the OC in OC-Zn^{2+} complex. Wang et al. (2004) [8] 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). The reason for the appearance 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 favourable to the interaction with anionic constituents of microbial cells and showed higher antimicrobial activity [5, 6, 8]. The results of this work about the DI and DS on soybean plant artificially inoculated with *C.truncatum* and treated with OC and OC-Zn^{2+} also considerably contributed for a new finding of an alternative agrochemical.

4. CONCLUSIONS

The OC with Mw ~5000 and OC-Zn^{2+} (1/0.5 in mol ratio) complex were successfully prepared. The structural properties of OC and OC-Zn^{2+} complex, in which Zn^{2+} ions interacted with the OC molecules through coordinate bonds were characterized by UV-Vis, FTIR, and XRD techniques. The prepared OC-Zn^{2+} complex exhibited strong antifungal activity against anthracnose disease causing by *C. truncatum* on soybean plants. Therefore, OC-Zn^{2+} complex, as an alternative bio-fungicide, have a potential for application in controlling anthracnose disease in crops. However, field experiments on the antifungal effect of OC-Zn^{2+} complexes should be further carried out.

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CẮT MẠCH BỨC XẠ CHẾ TẠO OLIGOCHITOSAN, PHỨC OLIGOCHITOSAN-Zn^{2+} VÀ HIỆU ỨNG KÍCH KHÁNG BỆNH THÁN THƯ TRÊN CÂY ĐẬU NÀNH

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Tóm tắt

Đặt vấn đề: Thán thư là một trong những bệnh gây thiệt hại nghiêm trọng trong canh tác đậu tương. Có một số hạn chế để kiểm soát bệnh này từ việc sử dụng thuốc hóa học truyền thống trong sản xuất nông nghiệp hiện đại. Những năm gần đây, oligochitosan (OC) và phức hợp OC-Zn^{2+} đã được đánh giá có hiệu ứng kháng nấm bệnh hiệu quả. Tuy nhiên, việc nghiên cứu phương pháp chế tạo và công thức tối ưu để sản xuất và ứng dụng chúng vào thực tiễn vẫn đang được khuyến khích tích cực.

Mục tiêu: Nghiên cứu chế tạo OC có khối lượng phân tử nhỏ nhưng đảm bảo cấu trúc hóa học, chế tạo phức hợp OC-Zn^{2+} có hiệu ứng kháng bệnh cao, đồng thời minh chứng các đặc trưng tính chất và đánh giá hiệu quả kháng bệnh thán thư của chế phẩm trên cây đậu tương.

Phương pháp: Dung dịch OC 4% được bằng phương pháp cắt mạch bức xạ bởi tia gamma kết hợp với một lượng nhỏ H_2O_2. Phức hợp OC-Zn^{2+} được chế tạo bằng cách khuấy trộn ở điều kiện thường trong 3 giờ. Đặc trưng tính chất của chế phẩm được xác định bằng các kỹ thuật GPC, UV-Vis, XRD và FT-IR. Hiệu quả chống lại bệnh thán trên cây đậu nành được thực hiện trong nhà màng, đánh giá tỷ lệ bệnh và chỉ số bệnh sau khi phun chế phẩm và phun bào tử nấm để gây nhiễm bệnh nhân.

Kết quả: OC chế tạo được có cấu trúc hóa học gần như không thay đổi so với chitosan ban đầu. Trong dung dịch với tỷ lệ phần mol 1/0,5 đã có sự tương tác giữa Zn^{2+} ion với các phân tử OC tạo thành các dạng phức hợp OC-Zn^{2+}. Phức hợp OC-Zn^{2+} có khả năng kháng bệnh bệnh thán thư trên cây đậu tương cao hơn so với OC. Tỷ lệ bệnh (DI) là 52,2% và 33,3% (so với đối chứng 92%); chỉ số bệnh (DS) là 35,7% và 13,8% (so với đối chứng 79,9%) tương ứng đối với cây xử lý OC và OC-Zn^{2+}.

Kết luận: OC với Mw ~5000 và phức hợp OC-Zn^{2+} với tỷ lệ mol là 1/0,5 đã được chế tạo thành công. Hiệu quả khích kháng bệnh bệnh thán thư trên đậu tương của OC, OC-Zn^{2+} đã được khảo sát và kết quả cho thấy phức hợp OC-Zn^{2+} có hiệu ứng cao hơn đáng kể so với OC. Phức OC-Zn^{2+} chế tạo từ OC cắt mạch bức xạ có tiềm năng sản xuất quy mô lớn và triển khai ứng dụng trong canh tác nông nghiệp như chất kích kháng bệnh hiệu quả.

Từ khóa: *Oligochitosan, kẽm, bệnh thán thư, bức xạ tia gamma.*