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CHITINASE-INDUCED RESISTANCE AGAINST *Neoscytalidium dimidiatum* ON DRAGON TREES: THE EFFECT OF OLIGOCHITOSAN PREPARED BY THE HETEROGENEOUS DEGRADATION OF CHITOSAN WITH H_2O_2 UNDER HYDROTHERMAL CONDITIONS

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Abstract

The heterogeneous degradation of chitosan with 5 % H_2O_2 under hydrothermal conditions (t = 75 minutes) was studied. The molecular weight of chitosan oligomer is 5 kDa. The degraded chitosan was characterized by gel permeation chromatography (GPC), Fourier transform infrared spectroscopy (FT-IR). Oligochitosan (OCT) can regulate in plant defense responses in many aspects. In this study when treated with oligochitosan (OCT 150 ppm), the dragon trees increased the activities of chitinase resistance against *Neoscytalidium dimidiatum* in white spot disease. The result suggested the role of triggering a mechanism of OCT for pathogen inhibition and disease control.

Keywords. Oligochitosan, chitinase, *Neoscytalidium dimidiatum*.

1. INTRODUCTION

Chitosan is obtained by partial deacetylation of chitin. Chitin is an abundant natural biopolymer which presents in the shells of some shellfish (shrimp, crab, and squid) composed about 20-30 %. Chitosan has been reported as a promising to control postharvest diseases and increase in plant defense responses. Chitosan is a polysaccharide with a chemical structure of polyβ-(1/4) N-acetyl-D-glucosamine (Fig. 1).

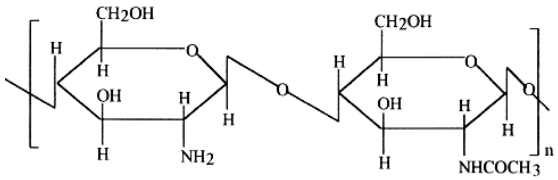

Figure 1: Chemical structure of chitosan

Chitosan has a high molecular weight which leads to insolubility at neutral pH with high viscosity. These difficulties have limited applicability of chitosan in the field of food, cosmetic, health and agriculture. Along with the purpose of solving the limitations of chitosan, researchers have also focused on the transformation of chitosan to oligosaccharide called oligochitosan (OCT) which are composed of 3 to 30 original glucosamines. The recent studies of oligochitosan have considerable attentions such as: water-soluble products and the special properties of chitosan as antifungal, antibacterial, antioxidative activity [3, 4]. OCT is promising environmentally friendly biomaterials for applications in many fields.

Several methods have been tried to prepare OCT such as: (1) Chemical treatment using oxidizing agents (H_2O_2, …) is an easy and low cost method but chemical waste and reproducibility are the main problems; (2) Biological treatment using enzymes is an effective way but it requires multi-steps, long time, enzyme preparation and purification of the product; (3) Irradiated treatment using radiation (gamma, neutron, electron beam ...) is a simple and environmental friendly process but high cost [1]. The objective of this study was to prepare OCT by H_2O_2 under hydrothermal conditions and to identify the capacity of pilot production.

Recently it was found, that molecular weight (M_w) comprised between 5,000 and 10,000 Da of OCT is one kind of potential signal for eliciting defense response in plants [4]. The elicitor is

produced when compounds received activation signal of microorganisms or synthesized from other compounds. Once OCT signals impact on cell cytoplasm, these signals transmit quickly and develop into a network of complex signals. Oligochitosan has the generation of phytoalexins (chitinase and glucanase) against fungal diseases, these enzymes provoke the disruption of the cell walls of fungi. This destroys the fungi, the increase of these enzymes influents the existence of fungal diseases. Recent findings and concepts provide the chitinase effect on plant defense responses [4-8].

The dragon trees ("pitahaya or pitaya") are grown in many countries in Southeast Asia such as Vietnam, Malaysia, Thailand, The Philippines, Indonesia. In Vietnam, the dragon fruit which has high economic value has been planted in several provinces such as Binh Thuan, Tien Giang, Long An... The white spot disease is caused by *Neoscytalidium dimidiatum* on dragon trees [9]. At present, the development of alternative stratergies to reduce the employment of classical chemical inputs for protection against diseases on dragon trees is becoming a necessity. Other objectives of this study are to highlight the usage of OCT as an elicitor and results from the chitinase-induced resistance against *Neoscytalidium dimidiatum* on dragon tress to determine their effective use on dragon trees as a biopesticide.

2. MATERIALS AND METHODS

2.1. Reagents

Chitosan (85% deacetylated, M_w = 20,312 Da) and standard chitosan were purchased from Sigma-Aldrich. *N*-acetylglucosamin (GlcNAc) and 3,5 dinitrosalicylic acid (DNS) were purchased from Sigma-Aldrich. Potato Dextrose Agar (PDA) medium and *Neoscytalidium dimidiatum* were provided from Plant Protection Department - Agriculture and Forest University. The molecular weight (M_w) of degraded chitosan was determined by an Agilent1100 gel permeation chromatography (GPC) (Agilent Technologies, USA) with detector RI G1362A and the columns Ultrahydrogel model 250 and 500 from Waters (USA). The standards for calibration of the columns were pullulan (M_w: 780–380×10^3). The eluent was aqueous solution 0.25 M CH_3COOH/0.25M CH_3COONa with the flow rate of 1.0 ml min^{-1} and temperature 30^oC. The chitosan sample concentration was 0.1 % (w/v). The IR spectrum was taken on FT-IR 8400S spectrometer (Shimadzu, Japan) [1, 2].

2.2. Heterogeneous degradation of chitosan with H_2O_2 under hydrothermal conditions

Chitosan (10 g) was impregnated in 10 ml of 5 % (wt%) H_2O_2 aqueous solution for 24 hours, then the autoclave was used for steaming the chitosan under T = 65 ^oC, p = 1.05 atm at 30 mins, 45 mins, 60 mins, 75 mins and 90 mins, respectively. After the reaction, the collected solid was washed 2 times with distilled water and then dried at 55 ^oC in vacuum. OCT was dissolved in lactic acid solution 5 %, the ratio of OCT: lactic acid solution = 1:10. This solution was diluted with distilled water at the rate of 1:100.

2.3. Characterization of antifungal effect of OCT against *Neoscytalidium dimidiatum*

OCT solution concentrations were 130 ppm; 150 ppm; 170 ppm. For antifungal assay, OCT solutions were added into PDA medium at concentrations 130 ppm, 150 ppm and 170 ppm, respectively in sterile culture plates (90 mm diameter). The petri dishes were incubated at 34-37 °C, RH = 75-80 %. The mycelial radial growth measurements were determined after 2, 4 and 6 days, respectively. For each concentration, three replicates were used.

2.4. Enzyme chitinase activity assay on dragon trees

Chitinase activity was determined using stem extracts (~20 cm) before and after OCT injections (2 days, 4 days and 6 days, respectively). An amount (200 g) of each sample was stored at cold temperatures ranging 0-5 ^oC. The samples were taken from 3 different locations on the same tree [8].

The homogenates (50 g stem extract with 50 g distilled water) were centrifuged and the supernatants were used for enzyme assays. The chitinase content was identified by measuring the rate of N-acetyl-glucosamine release from chitin (crab shells, Sigma). The reaction mixture contained 10 mg/ml of chitin in pH 5.0 and 2 ml of enzymatic extract. After 3 h of incubation, the reaction was stopped by heating in boiling water for 5 mins, then the reaction was cooled down to ambient temperature. 2 ml of NaOH 2 M aqueous solution was added to the each mixture and drew 3 ml per tube. Each tube was added 1 ml of DNS and heated in boiling water for 5 mins then it was heated at 30^oC for 30 mins. The absorbance at 540 nm of the mixture was mesured by UV-visible spectroscopy using a Varian 5000 spectrophotometer at University

of Science Ho Chi Minh city. The results were calculated as follows:

$$HT = \frac{X.k.V}{v.t}$$

Note: HT is the chitinase activity (IU/g); X is the amount of reducing sugar produced (μg/g); k is the coefficient of dilution; V is the total volume of the reaction solution (ml); v is the volume of enzymatic extract (ml); t is the reaction time (min).

2.5. Field condition

+ Treated lot: OCT 150 ppm was sprayed to 800 ml/pillar. Each experimental lot consisted of 40 pillars

+ Control lot: Water was sprayed.

+ After 24 hours, 10^2 CFU/ml of *Neoscytalidium dimidiatum* was spayed to 300 ml/pillar.

+ The Rate Disease and Index Disease were controlled after 2 days and 6 days of the pathogen invasion.

+ The Rate Disease (RD) was calculated using the formula (5 pillars were selected):

$$RD\ (\%) = \frac{\text{Total number of infected branches}}{\text{Total number of branches in 5 pillars}} \text{x } 100$$

+ The Index Disease (ID) was calculated using the formula (5 pillars were selected):

$$ID\ (\%) = \frac{(N_1.1) + (N_2*2) + \ldots\ldots + (N_5*5)}{5N} \text{ x } 100$$

where N is the total number of branches in 5 pillars.

N_1, N_2, N_3, N_4, N_5 is the infected branche at each level 1, 2, 3, 4, 5, respectively.

Pathogen levels were divided:

Level 1: 0-10 % of infected branch length

Level 2: 11-20 % of infected branch length

Level 3: 21-30 % of infected branch length

Level 4: 31-40 % infected branch length

Level 5: > 41 % of infected branch length.

These experiments were conducted at Ham Thuan Nam District, Binh Thuan province from 09 to 11/2013

3. RESULTS AND DISCUSSION

3.1. Heterogeneous degradation of chitosan with H_2O_2 under hydrothermal conditions

The study results showed that the reaction time and M_w of the OCT (Table 1) correlated inversely proportional. After 60 minutes, M_w decreased from 20,312 Da to 7,768 Da and for 75 minutes, the M_w of OCT is 5,038 Da. This reaction was continued but the molecular weight was not reduced significantly. Thus, the reaction time of degreaded chitosan under hydrothermal condition is the most effective for 75 mins. The polydispersity index (PI) ranged from 1.33 to 1.71. This shows that the degradation of chitosan create oligochitosan with the M_w distribution in a narrow range.

Table 1: M_w, M_n, PI of heterogeneous degradation of chitosan with H_2O_2 under hydrothermal conditions

No	Time (mins)	Mw (Da)	Mn (Da)	PI = Mw/Mn	DDA (%)
1	After impregnation in H_2O_2	20,312	15,263	1.33	85.8
2	30 (in autoclave)	13,370	8,228	1.62	85.0
3	45 (in autoclave)	10,475	6,127	1.71	82.4
4	60 (in autoclave)	7,768	4,735	1.64	82.1
5	75 (in autoclave)	5,038	3,324	1.52	81.5
6	90 (in autoclave)	4,645	2,865	1.62	81.0

The molecular weight M_w of degraded chitosan decreased remarkably with increasing the hydrothermal time. The M_w of degraded chitosan reduced slightly when the reaction time is greater than 75 min. The equation of relation betwwen M_w and the reaction time is $y = 21478e^{-0.017x}$ (R^2 = 0.9802) (figure 1).

The hydrothermal time and H_2O_2 agent led to the reduction in molecular weight of degraded chitosans. FTIR spectra revealed that the characteristic functional groups in chitosan as hydroxyl groups (OH) in the wavelength ranging from 3430-3457 cm^{-1} and the amide group band I and free amino groups ranging from 1664-1667 cm^{-1} did not change

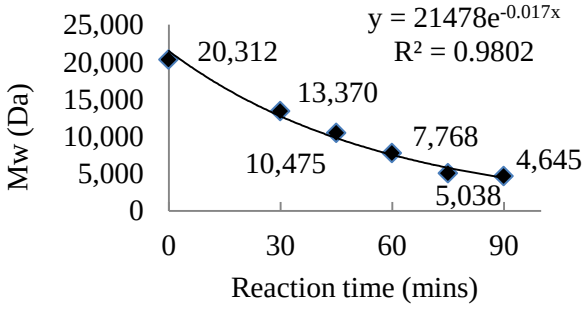

Figure 1: Time effect on the molecular weight (M_w) of degraded chitosan

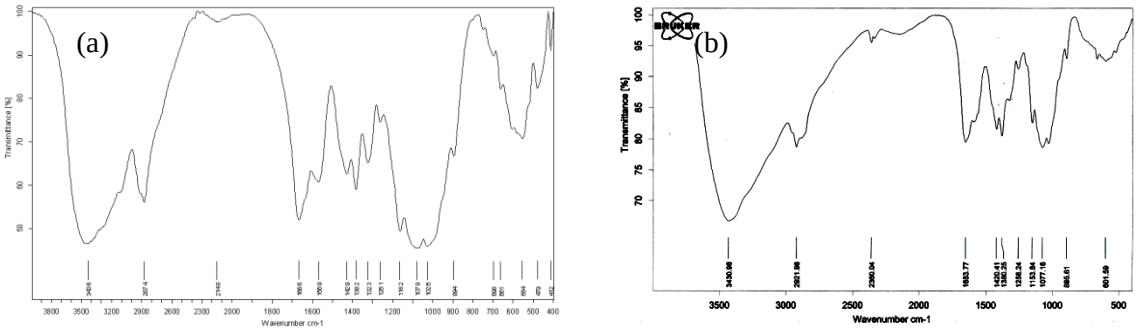

Figure 2: (a) IR spectrum of standard chitosan (Sigma); (b) IR spectrum of OCT (M_w = 5,038 Da, t = 75 min)

compared with the standard sample (figure 2). The degradation of chitosan with H_2O_2 under hydrothermal condition do not affect the functional groups of chitosan.

3.2. *In vitro* antifungal assay against *Neoscytalidium dimidiatum*

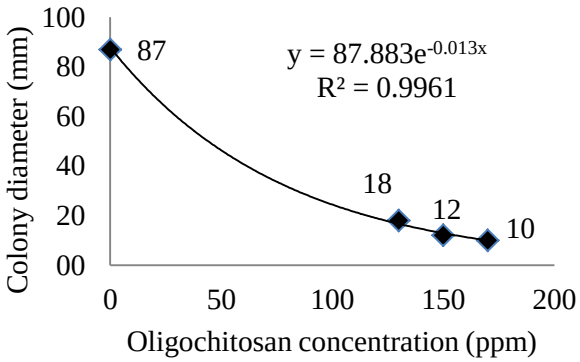

Figure 3: The diameter of colonies depends on the concentrations of OCT after 6 days of culture

Figure 3 showed that after 6 days of culture and effective testing, the antifungal effect against *Neoscytalidium dimidiatum* was statistically significant at α = 0.05 significance level. The fungal resistance increased with increasing the OCT concentration. At OCT contents (130; 150; 170 ppm) it was clear that the antifungal activity increased and OCT 170 ppm concentration exhibited antifungal potency against *Neoscytalidium dimidiatum* with IC_{50} = 51.5 ppm. The equation of antifungal activity against *Neoscytalidium dimidiatum* of OCT was $y = 8.7883e^{-0.013x}$ (R^2 = 0.9961).

3.3. Oligochitosan effect to the chitinase activity on the dragon trees

The dragon trees generate an amount of enzyme chitinase, the chitinase activity ranged from 17.6 to 24.4 UI/g. When OCT was sprayed on the dragon trees, the chitinase activity increased. After premier injection, the chitinase content increased from 24.4 to 34.9 UI/g. After *Neoscytalidium dimidiatum* infection, the chitinase activity dropped significantly on dragon trees, this enzyme produced was used in the mechanism of elicitor [9] so the chitinase enzyme activity dropped sharply from 5.6 to 6.4 UI/g. However, the treated samples decreased rate and index disease (Section 3.3), the lesion almost healed. After 4 and 6 days, the infectious lesions of 2 treated samples have been controlled, the amount of chitinase produced and continued to increase to 19.1 UI/g, and for the control samples, chitinase activity increased slightly because almost trees were still sick (figure 5). The study results showed that OCT effect of the chitinase activity was born by the trees dragon.

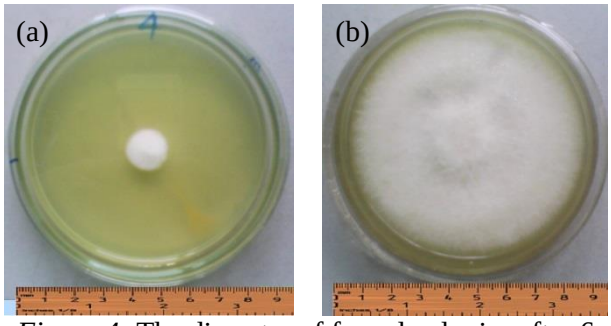

Figure 4: The diameter of fungal colonies after 6 days of culture: (a) OTS 150 ppm; (b) Control

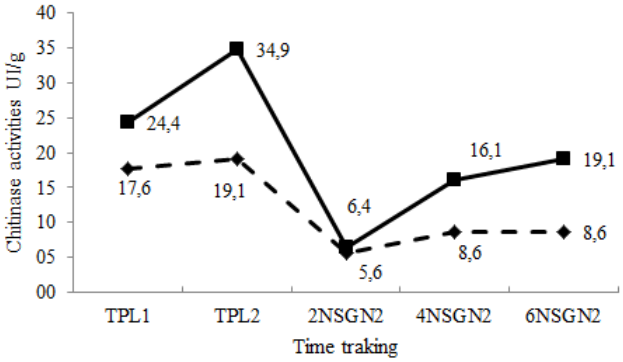

Figure 5: Chitinase activity changes according to the time

3.4. Oligochitosan effect against *Neoscytalidium dimidiatum* on the dragon trees

Oligochitosan effect against the white spot on the dragon trees in Binh Thuan province was shown in table 2.

The rate and index disease of OCT 150 ppm were at low levels compared to controls ($\alpha = 0.05$). After 2 days of injection of white spot disease, the rate disease was 6.54, control samples have high infection rate of 13.10 %. After 6 days of the infectious disease, the rate dropped sharply for treated samples (2.08 %), while the control samples continued to increase and spread faster rate (18.85 %). Similarly, the disease after 6 days of treatment OCT was very low infectivity (0.46 %) compared to controls (5.85 %). OCT 150 ppm active elicitation effect to white spot. This helps plants recover faster.

Table 2: Rate disease (%) and Index disease (%) on the dragon trees

Objects	Rate disease (%)		
	TP	S2NGN	S6NGN
Control	3.54±1.29a	13.10±4.28a	18.85±3.84a
OCT 150 ppm	4.11±0.33a	6.54± 1.99b	2.08±0.59b
LSD 0.05	0.51	1.15	0.93
	Index disease (%)		
Control	0.89±0.36a	3.87±1.44a	5.85±1.36a
OCT 150 ppm	0.99±0.37a	1.77±0.60b	0.46±0.15b
LSD 0.05	0.14	0.40	0.93

Note: TP: before injection; S2NGN: after 2 days of the injection of fungal invasion; S6NGN: after 6 days of the injection of fungal invasion control; OCT: oligochitosan. Means followed by the same letter in each column are not significantly different according to Duncan's Multiple Range Test at 5 % level.

4. CONCLUSION

Oligochitosan (Mw ~ 5,000 Da) has been prepared with H_2O_2 5 % under hydrothermal conditions (T = 75 ^oC). Oligochitosan (150 ppm) active chitinase stimulation on the dragon trees that are resistant to *Neoscytalidium dimidiatum*.

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