Synthesized nanochitosan induced rice chitinase isozyme expression; application in brown planthopper (BPH) control

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ABSTRACT

Chitosan nanoparticles were prepared by ionic gelation of a chitosan solution with anionic sodium tripolyphosphate (TPP). The XRD pattern of chitosan exhibited broad diffraction peaks at an angle 2θ of 21º, which were the characteristic peaks of chitosan. The FESEM image confirmed that the nanoparticle products were spherical in shape with a size of 100-200 nm. The FTIR spectrum of chitosan exhibited O-H, C-H, C-N, C-O-C band stretching and N-H bending, which were in good agreement with what has previously been reported**.** Chitosan nanoparticles were applied for rice growth and protect brown planthoppers. The concentration of chitosan nanoparticles at 10, 50 and 100 ppm promoted rice growth when compared to the control, while rice seedlings did not grow when chitosan nanoparticles at a concentration of 500 and 1000 ppm were applied. Protein content and chitinase activity in rice treated with nanochitosan varied depending on the chitosan concentration and rice growing time. Total protein content was up to 23.66 fold, while chitinase enzyme activity increased to 3.31 fold when compared to the control. Four isoforms were presented in all plant tissue after two weeks following nanochitosan induction. One isoform (18 kDa) was constitutively presented in the control, while the other three isoforms (12 kDa, 24 kDa, 76 kDa) were strongly induced by nanochitosan. Chitinase isoform expression in rice seedlings may be involved in rice against to pathogen and insect. Synthesised nanochitosan successfully induced chitinase isoform expression in rice.

Keywords: chitinase, nanochitosan, rice, isozyme, brown planthopper

INTRODUCTION

Chitinase is defensive enzyme that catalyses the β-1,4 glycoside bond present in chitin (Kirubakaran, S.I. and Sakthivel, N., 2007). Chitin is a primary structure of bacterial and fungal cell walls and insect skeletons. Chitinase enzymes are classified based on their isolation, structural and functional characterisation. Most of the chitinase enzymes in plants belong to family 19, while prokaryotic and eukaryotic belong to the group in family 18 (Sharma, N. *et al*., 2011). Recently, several reports found that chitinase enzymes isolated from plants such as plant latex (Sytwala, S. 2015), rice (Arora, N. *et al*., 2015), *Capsicum annuum* (Kim, D.S. *et al*., 2015) and bacteria such as *Streptomyces* sp. (Karthik, N., 2015), thermophilic marine bacterium (Yang, S. *et al*., 2016), *Bacillus licheniformis* (Laribi-Habchi, H. *et al*., 2015) and fungi (Karthik, N. *et al*., 2014) indicate the inhibition of the growth of plant pathogens, while the expression of cloned chitinase in transgenic plants has provided

evidence for their role in plant disease protection. Therefore, high accumulation of chitinase enzymes in plants is essential for protection against plant pathogens.

However, chitinase enzymes in plants are constitutively expressed at low levels. Inducing expression of chitinase enzymes by abiotic (ethylene, salicylic acid, salt solution, ozone, UV light) and biotic stress (fungi, bacteria, viruses, viroids, chitin and chitosan) have been reported (Punja, Z. *et al*., 1993).

Chitosan is one of the elicitors induced for chitinase expression in plants. It is a natural polymer derived from the deacetylation of chitin. Chitin is obtained from the skeletons of insects, crustaceans (shrimp, crab) and some fungi. Chitosan structures are composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). Chitosan is soluble in diluted acids such as acetic and formic acid (Ravi kumar, M.N.V. et al., 2000). It has a potential role in agriculture with regard to control plant diseases in the form of antivirals, antibacterials and antifungals (Hadrami, A.E.L. *et al*., 2010). A chitosan solution for use against the rice bacterial brown stripe pathogen *Acidovorax avenae* subsp.*avenae* RS-1 has been reported (Yang, C. et al., 2014).

The application of chitosan for rice growth promotion and pathogen protection has been reported, e.g., chitosan induction as a defence response against *Pyricularis grisea* (Rodriguez, A.T. *et al*., 2007). Liu, H. et al., (2012) reported chitosan as a defence against the rice sheath blight pathogen *Rhizoctonia solani*. Interestingly, chitosan showed a positive effect in terms of increasing rice yield and inhibiting the presence of brown backed rice planthoppers (BPH) (Toan, N.V. *et al*., 2013)

However, the poor solubility of chitosan is a major limiting factor in its utilisation. To clarify this point, in the present study, nanochitosan will be synthesized using an ionic gelation method. The nanochitosan produced using this method is stable, non-toxic, organic and solvent free. Electrostatic interaction of newly synthesized nanochitosan occurs between amino groups and Tripolyphosphate (TPP). We expected that nanochitosan would induce chitinase expression in rice, thus protecting against brown planthoppers and rice pathogens.

METHODOLOGY

Rice planting

Rice cultivar Phitsanulok 2 (*Oryza saltiva* cv. indica) was obtained from the Phitsanulok Rice Research Center, Phitsanulok, Thailand. Rice seeds were soaked in water for 48 h; germinated seeds were transferred for growth in a plastic box containing soil and were allowed to grow for two week. The plastic box containing the rice seedlings was soaked in a synthesized nanochitosan solution at a concentration of 10 ppm, 50 ppm, 100 ppm, 500 ppm and 1000 ppm, respectively. The control was soaked in water. Rice seedlings were sampled at one week and two weeks-old for chitinase enzyme assay and protein content.

Preparation of chitosan nanoparticles

The chitosan nanoparticles were prepared by the ionic gelation of chitosan powder, as noted in a previous reports (Calvo, P. *et al*., 1997; Zhang, H.I. *et al*., 2009) Firstly, the chitosan solution was prepared by dissolving chitosan powder in acetic acid (2% v/v) at different concentrations of 0.1, 0.3, 0.5, 1.0 and 3.0 mg/ml. Then, 5 mL of 6.0 mg/mL TPP solution was added, drop-for-drop, into a 20 ml chitosan solution under magnetic stirring in order to disperse the droplets. After stirring the mixture for 15 min, nanoparticles formed via the interaction between the negative groups of TPP and the positively charged amino groups of chitosan (Calvo, P. *et al*., 1997) . Finally, the synthesized chitosan nanoparticles were collected from the suspension using a freeze dryer.

Chitosan nanoparticle characterisation

The structural analysis of the prepared samples was conducted by X-ray powder diffraction (XRD; Philip X' Pert PRO PW 3719) using Cu K_α radiation (k = 1.5406 A°). In order to measure powder diffraction using the XRD method, the chitosan powder was placed in the sample holder ring and pressed slightly with a glass slide. The morphological characteristics (size, shape and appearance) of chitosan nanoparticles were observed by field-emission scanning electron microscopy (FESEM; JSM-6335F, JEOL). A specimen was prepared from the dispersion of chitosan nanoparticles in ethanol and homogenized with a vibra-cell sonicator (Sonics VCX 750) for 15 min. Then, the resultant suspensions were applied, drop-for-drop, onto an Al stub, followed by drying under sunlight for 20 min. Functional group confirmations were assessed by Fourier transform infrared spectrometer (FTIR; Bruker, Model Tenser 27). The FTIR specimen was obtained by pressing samples in KBr pellets.

ChitinaseEnzymeextraction

Rice plants were ground in an ice-cold mortar with liquid nitrogen and suspended in an acetate buffer pH5. Crude homogenates were centrifuged at 14000 rpm for 30 min at 4ºC and the supernatant fraction was kept frozen at -20ºC. Protein content were determined by the Bradford method using a bovine serum albumin (BSA) as standard.

Chitinase activity assay

Standard curve of N-acetylglucosamine

1 mg/ml of N-acetylglucosamine solution was separately added to a test tube at 10, 20, 30, 40 and 50 µl, respectively. Then, 200 µl of 0.8 M boric acid (pH 10.2) was added; then, water was adjusted to make a total volume of 1.20 ml. The incubation was conducted in boiling water for 3 min and then allowed to cool. Then, 3 ml of p-dimethyl amino benzaldehyde (DMAB) was added to test tube and incubated at 37ºC for 20 min. A purple solution resulted and was measured at 585 nm using a spectrophotometer (Cecil CE1010). A standard curve was obtained between A_{585} versus the amount of N-acetylglucosamine.

Chitinase assay

Colloidal chitin was used as a substrate for assaying chitinase activity; 0.01 g of colloidal chitin in a 0.1 M sodium acetate buffer (pH 5) and 900 µl of enzyme solution was incubated at 37ºC for 30 min. After incubation at 30 min, the reaction was terminated by centrifuge at 4000 rpm for 10 min. The 500 µl of supernatant was collected and added to the test tube. Subsequently, 100 µl of 0.8 M boric acid (pH 10.2) was added; the mixture was boiled in a water bath for 3 min and allowed to cool. Then, 3 ml of DMAB solution was added and incubated at 37ºC. The product was measured at 585 nm using a spectrophotometer.

One unit of enzyme activity was defined as the amount of enzyme that catalysed the release of 1 µmol of N-acetylglucosamine per 1 ml in 60 min.

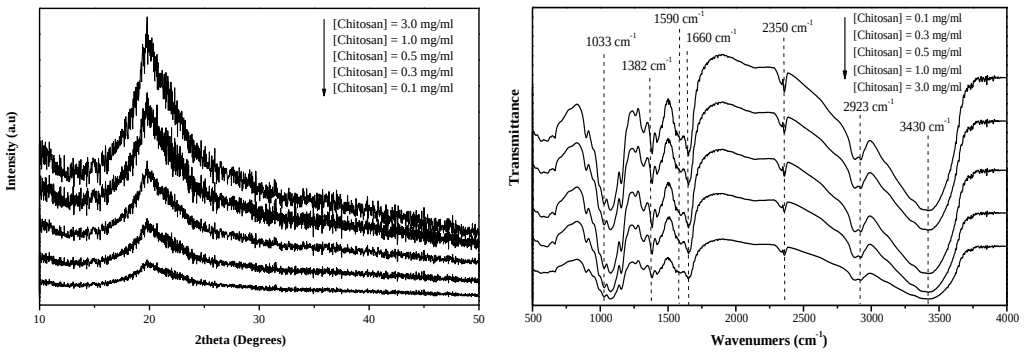
Chitinase activity staining in native polyacrylamide gel (PAGE)

Native-PAGE for chitinase was performed in a 12.5% polyacrylamide gel containing colloidal chitin as substrate; 10 µg of crude enzyme extracts were subjected to electrophoresis. Following gel electrophoresis, the gel was equilibrated in a 100 mM sodium acetate buffer containing 1% Triton X-100 at 37ºC for 22 h. The gel was then washed with distilled water and fixed with 40% ethanol and 10% acetic acid for 30 min. The clear zone of chitinase isoforms was visualized following Coomassie Blue G staining.

RESULTS AND DISCUSSION

The X-ray diffraction patterns of nanochitosan

X-ray diffraction patterns were performed in order to study the phase composition of nanochitosan. The obtained XRD patterns for chitosan nanoparticles at different concentrations are shown in Figure 1a. It was found that all samples presented similar characteristic peaks of $2\theta = 20.1°$, which was in agreement with previous reports (Harish Prashanth, K.V. *et al.*, 2002; Wang, X. *et al.*, 2004). However, when increasing the chitosan concentration, the XRD peak intensity also increased, with the 3.0 mg/ml having the highest intensity. The broadening of the peak was due to the amorphous nature of the chitosan polymer (Rhim, J.W. *et al.*, 2006); the peak became broader when decreasing the chitosan concentration. Additionally, no impurity peaks were found in the XRD pattern.



(a) (**b**)

Figure 1 Chitosan characterisation: (**a**) XRD pattern of chitosan at different concentrations; (**b**) FTIR spectra of chitosan nanoparticles at different concentrations.

Fourier transform infrared spectrometer (FTIR)

FTIR spectroscopy was used to obtain the chemical groups of nanochitosan. Figure 1b shows the FTIR spectra of chitosan nanoparticles. The broad peak at 3430 cm^{-1} is assigned to the N-H stretching vibration, while stretching vibrations of 3600 cm^{-1} could not be observed distinctly due to overlapping with broad peak of –N-H and –OH .The vibrational band observed around 2923 cm^{-1} was the result of the typical C-H stretching from alkyl groups and the peaks between 1660 and 1590 cm^{-1} were due to N-H bending. The peak observed around 1033 cm^{-1} indicated C-O-C bending in chitosan, while another broad peak at 2350 cm^{-1} was caused by CO_2 in the air. In addition, absorption peaks at 1382 cm^{-1} was shown, which had been reported as C-N stretching (amide III) (Peniche, C. *et al*., 1999). Moreover, the spectra show that with a decrease in chitosan concentration, the peak intensity became more intense than those of high concentrations, owing to pellet thickness. Since high chitosan concentrations caused difficulties in obtaining clear pellets, they exhibited low transparency to the incoming light source.

Field-emission scanning electron microscopy (FESEM)

The morphology and particle size of synthesized chitosan nanoparticles were analysed using the FESEM technique. Figure 2(a) shows that nanoparticle products were spherical in shape with a size of 50-100 nm. A similar morphology was also observed for 0.3 mg/ml chitosan (Figure 2b). The sizes of these nanoparticles were consistent with those determined from 0.1 mg/ml chitosan. Upon increasing the chitosan concentration, however, spherical primary particles became more aggregated, as seen in Figure 2(c), (d) and (e).

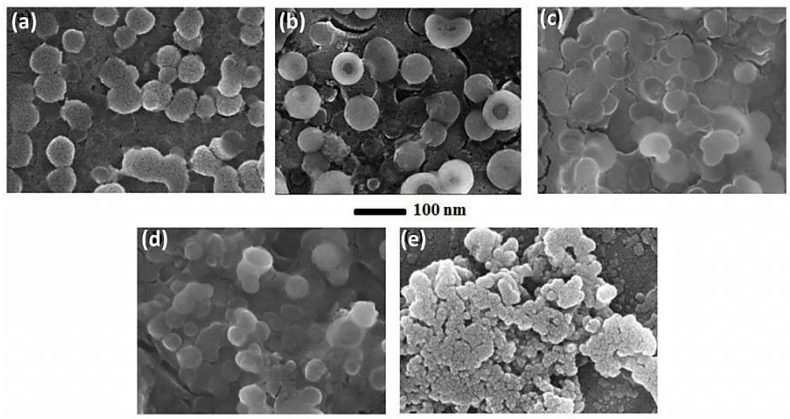
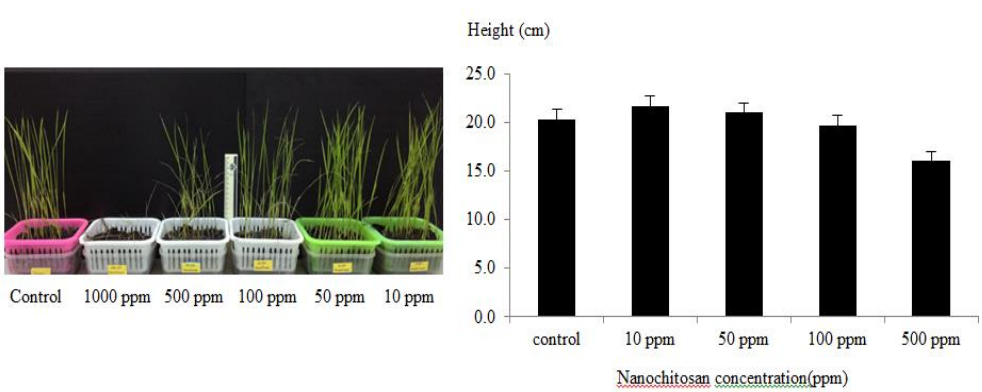


Figure 2 Field-emission scanning electron microscopy (FESEM) at different concentrations: (a) 0.1 mg/ml; (b) 0.3 mg/ml; (c) 0.5 mg/ml; (d) 1.0 mg/ml; (e) 3.0 mg/ml.

Effect of nanochitosan on rice growth

Rice seedlings were grown in nanochitosan at different concentrations of 10 ppm, 50 ppm, 100 ppm, 500 ppm and 1000 ppm, respectively. Nanochitosan at concentrations of 100 ppm, 50 ppm and 10 ppm showed activated plant growth compared to the control (Figure 3). Rice seedlings did not survive in a nanochitosan solution at concentrations of 500 ppm and 1000 ppm for a period of two weeks. Many reports support this result, e.g., Chamnanmanoontham, N. *et al*., 2015, who used 40 ppm of chitosan solution and found it to significantly enhance rice growth in terms of leaf and root growth when compared to the control. Similarly, results showed that chitosan stimulated plant height when cv.Suphanburi rice seeds were soaked in chitosan at a concentration of 80 ppm for 4-5 hours (Boonlertnirun, S. *et al*., 2008).



(a) **(b)**

Figure 3 Rice seedlings growing (a) in a nanochitosan solution at different concentrations of 1000 ppm, 500 ppm, 100 ppm, 50 ppm and 10 ppm; (b) rice growth.

These results (**Figure 3**) indicate that chitosan contains a positive ionic that form an ionic bond with plant nutrients harbouring negative ions, resulting in the slowly released action of nutrients within the plant (Boonlertnirun, S. *et al*., 2008).

Protein concentration in rice seedlings treated with chitosan nanoparticles

This research also investigated protein concentrations in rice seedling samples. The amount of protein in rice treated nanochitosan can present an understating regarding the response system of rice to biotic stress. Crude protein from rice seedling samples treated with nanochitosan at different concentrations were extracted and quantified using the Bradford method. Protein concentrations increased following nanochitosan concentration application in rice (**Table 1**). The highest protein content was found in rice treated with nanochitosan at 500 ppm and 1000 ppm after one week and two weeks, respectively. Rice seedlings treated with nanochitosan at 10 ppm, 50 ppm and 100 ppm showed no difference in terms of protein concentration. However, protein concentrations in treated rice samples showed higher concentrations than those in the control. This may have been due to nanochitosan acting as an abiotic stress (drought, high salinity, cold and heat) in rice seedlings. All of abiotic stresses can adversely affect plant growth and productivity (Basu, S. *et al*., 2014; Singh, A .*et al*., 2015).

Table 1 Protein content, enzyme activity and the specific activity of chitinase extracted from rice treated with a nanochitosan solution at different concentrations.

Concentration	Protein content (µg)		Enzyme activity (unit)		Specific activity (unit/µg)	
	1 weeks	2 weeks	1 weeks	2 weeks	1 weeks	2 weeks
Control	115±20.71	120±28.28	1.13±0.07	0.95±0.09	0.0079± 0.020	0.0075±0.013
10 ppm	145±7.07	180±14.14	1.14±0.05	1.02±0.01	0.0070± 0.017	0.0067±0.023
50 ppm	180±0.00	205±21.21	1.16±0.04	1.00±0.07	0.0049±0.040	0.0064±0.074
100 ppm	170±14.14	280±14.14	1.42±0.09	1.37±0.05	0.0049±0.010	0.0079±0.028
500 ppm	220±0.00	355±35.36	2.01±0.04	1.42±0.23	0.0040±0.012	0.0091±0.014
1000 ppm	310±14.14	-	3.75±0.39	0.95±0.09	0.0079±0.013	0.0121±0.018

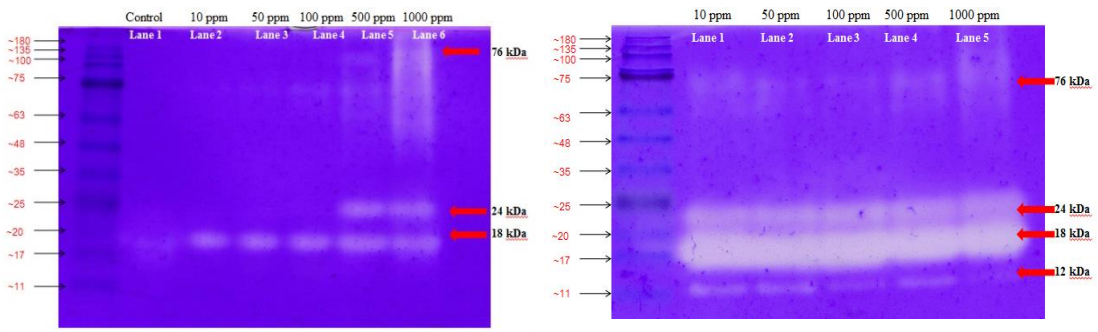
When rice plants are stressed by nanochitosan, the plant may adapt itself through the secretion of a defence protein. Most plants also protect themselves against biotic and abiotic stress by increasing protein secretion (Singh, A. *et al*., 2015; Chen, A. *et al*., 2014; Hashimoto, M. *et al*., 2004). For example, highly induced rice protein have been observed under abscisic acid, salt and drought stresses (Singh, A. *et al*., 2015). Heat shock proteins were up-regulated in rice seedlings in the case of a response to high temperature stress (Chen, A. *et al*., 2014). Novel rice PR10 protein was rapidly induced in roots by salt, drought stress and blast fungus infection (Hashimoto, M. *et al*., 2004). Recently, Chamnanmanoontham, N. *et al*., (2015) reported an increase in protein expression in rice leaves treated with chitosan compared to a control.

Chitinase activity in treated rice with different concentrations of chitosan nanoparticles

Enzymatic activity of chitinase was significantly increased in rice treated with nanochitosan when compared to the control. Increased chitinase activity was higher in two-week-old rice than one-week-old rice. Results showed that the chitinase activities were highest for 1000 ppm and 500 ppm of nanochitosan at 3.75 units and 2.01 units, respectively (**Table 1**)**.** Moderately increased enzyme activity was found in rice treated with nanochitosan at concentrations of 10 ppm, 50 ppm and 100 ppm, respectively. Previous reports noted chitinase as a defensive enzyme as induced by biotic and abiotic stress. Physical stimulation including wounding, ultraviolet-B radiation, osmotic shock, low temperature, water deficit or excess, as well as chemicals such as salicylic acid, chitin and chitosan were also involved in the induction of chitinase activity. Other responses include lignification, ion flux variations, cytoplasmic acidification, membrane depolarisation and protein phosphorylation (Hadrami, A.E. *et al*., 2010). Sena, A.P.A. *et al*., (2013) found that chitinase activities increased 72 hours after spraying rise with an *Epicoccum* sp. pathogen, while the induction of chitinase and β-1,3-glucanase activity were also observed in rice's response to a bacterial leaf blight pathogen (*Xanthomonas oryzae pv. oryzae*) (Babu, R.M. *et al*., 2003). Additionally, transgenic rice containing the chitinase gene showed enhanced resistance to sheath blight disease (Datta, K.A. *et al*., 2001). Additional evidence found that the highest activity of chitinase at 1.97-2.01 units was found in a resistant mango cultivar. In contrast, activity less than 0.010 was found in a highly susceptible cultivar (Ebrahim, S.K. *et al*., 2011). The activity of chitinase may be used as a criterion for predicting cultivars' resistance against pathogens. It is suggested that nanochitosan induces chitinase enzyme production, which has been shown to have a potential role against pathogens in rice. Therefore, chitinase isozyme will be investigated.

Chitinase isozyme expression through native gel electrophoresis

In this study, chitinase activity staining and its properties, including isozyme and molecular weight, were determined. Protein was extracted and then subjected to native gel electrophoresis containing a glycol chitin substrate. Chitinase enzymes will be catalysed substrate in gel, as a results clear zone on gel **(**Figure 4**)**. The clear zone represents the catalytic regions that occurred following the chitinase enzymes catalysed the substrate in polyacrylamide gel. Increasing clear zone intensity corresponded with nanochitosan concentration and planting time as shown in Figure 4. The results in lane 2 to lane 6 (Figure 4a) presents the clear zone following the treatment of rice with nanochitosan at a concentration of 10 ppm, 50ppm, 100 ppm, 500 ppm and 1000 ppm, compared to the control (lane 1), respectively.



(a) **(b)**

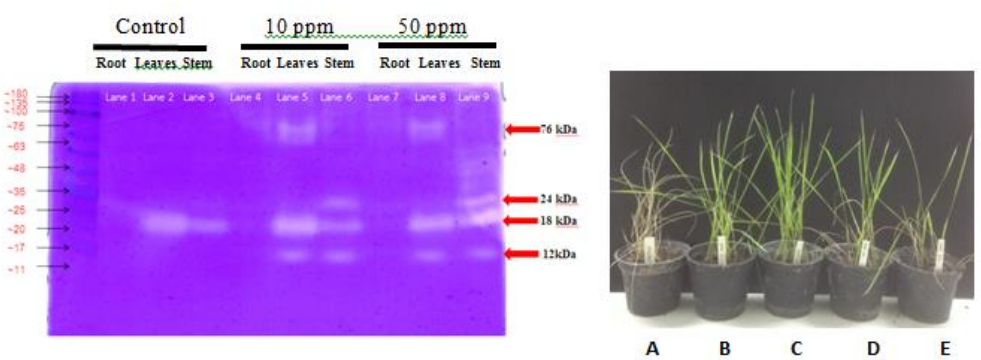
Figure 4 Activity staining of chitinase enzymes on polyacrylamide gel and electrophoresis incorporated with glycol chitin as substrate. The clear zone indicates that chitinase enzymes successfully catalysed the substrate on the gel below 37°C for a period of 22 h. Activity of rice chinase enzymes when rice was soaked with nanochitosan for (**a**) one week and (**b**) two-week-old rice.

The highest clear zone intensity was observed in rice response to nanochitosan concentrations at 500 ppm and 1000 ppm (lane 5,6). The results indicate that nanochitosan can induce chitinase production in rice plants. Following on, a high accumulation of chitinase in rice plants can activate a defensive system against pathogens and insects. In addition, the results of chitinase enzyme expression over a period of two weeks is shown in Figure 4b. The intensity of the clear zone during a period of two weeks was higher than the clear zone presented after one week. This suggests that after two weeks, rice may absorb nanochitosan for a longer term, subsequently inducing chitinase expression to protect plants from a stressful environment. However, the intensity of the clear zone showed no differences between lanes 1-5 in Figure 4b. The reason for this may have been because no difference changed of chitinase producing level from treated rice for 2 weeks.

In addition, the isoform pattern of chitinase enzymes was investigated in this study. The chitinase isoform correlation with pathogen against properties, indicating that each form of chitinase plays a different role in plant protection. For example, four chitinase isoforms (E, F, H1, G) presented in yam tubers. Chitinase E, F and H1 isoforms had high activity against pathogens, but chitinase isoform G did not (Arakane, Y. *et al*., 2000).

In this study, the chitinase isoform patterns observed in rice treated with nanochitosan varied depending on nanochitosan concentration and planting time. In lanes 2-6 (Figure 4a), rice treated with nanochitosan for one week showed the three isoforms 18 kDa, 24 kDa and 76 kDa at high concentrations of nanochitosan (at 500 ppm and 1000 ppm). Low levels of nanochitosan were shown at 10, 50 and 100 ppm; the control did not present the isoform pattern (lane 1-4). It is therefore indicated that nanochitosan can induce isoform expression of chitinase enzymes. Xie, Z.P. et al., (1999) reported that four chitinase isoforms were strongly induced in soybean nodules following mycorrhizal infection, while two isoforms were present in

the control. In other studies, two isoforms were detected in an untreated rice callus and an additional isoform was detected in a rice callus treated with O-carboxymethylchitin (Hirano, S. *et al*., 1994). However, at two weeks, the isozyme pattern of chitinase were presented as four isoforms at 12 kDa, 18 kDa, 24 kDa and 76 kDa, which were not detected in rice planted for one week (Figure 4b). These results suggested that rice planting time also induced chitinase enzyme production. To determine the localisation of chitinase isoforms in rice in this study, roots, leaves and the stem of rice seedlings were presented separately as shown in Figure 5a during the two-week period. Rice leaves presented three isoforms at molecular weights of 12 kDa,18 kDa and 76 kDa. Similarly, the stem also had three isoforms at 12 kDa, 18 kDa and 24 kDa, while rice roots did not present a clear zone; this indicates that chitinase isoforms are not found in rice roots. Moreover, two isoforms presented in rice leaves (76 kDa, 12 kDa) and differed from the control following nanochitosan induction. These isoforms may play a role in defending against pathogens that prefer to attack rice leaves. Furthermore, 24 kDa and 12 kDa isoforms were found in the stem after soaking rice in a nanochitosan solution, compared to the control. The 12 kDa and 24 kDa isoforms in the stem may protect rice plants from sucking stem insects such a brown planthoppers (BPH).



(a) (**b**)

Figure 5 Chitinase isozyme expression in (**a**) rice roots, leaves and the stem of rice seedlings treated with nanochitosan at 10ppm and 50 ppm, compared to the control; (**b**) rice plant after BPH attack for two weeks. Nanochitosan at different concentrations of A= control, B=10 ppm, C=50 ppm, D=100 ppm and E=500 ppm were applied to rice seedlings.

Application of rice treated with nanochitosan against brown planthoppers Rice plants were soaked in a nanochitosan solution at different concentrations of 10 ppm, 50 ppm, 100 ppm and 500 ppm for two weeks in a green house, while the control was soaked in water. Brown planthoppers were introduced to the rice plant for two weeks. The control plant did not survive, while rice treated with a nanochitosan solution at a concentration of 10 ppm, 50 ppm and 100 ppm showed significant growth following BPH attack. The results indicate that nanochitosan induces chitinase accumulation in rice against BPH (Figure 5b). In addition, rice treated with 500 ppm

nanochitosan did not promote rice growth due to a high concentration of nanochitosan.

CONCLUSIONS

Chitosan nanoparticles was successfully synthesized by TPP with the size of 100-200 nm and characterized using XRD, FESEM and FTIR.This study used nanochitosan in rice plants to protect against BPH attack. Nanochitosan not only promoted plant growth but also effectively induced total protein and chitinase activity in rice. Four chitinase isoforms were induced following the treatment of rice with nanochitosan; isoforms at a molecular weight of 12 kDa and 76 kDa may be involved in protecting against pathogenic attacks against rice leaves. The other isoforms at a molecular weight of 12 kDa and 24 kDa may also play a role in protecting against pathogenic attacks in stems of rice plants.

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