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Chitosan as a Biopesticide against Rice (*Oryza sativa*) Fungal Pathogens, *Pyricularia oryzae* and *Rhizoctonia solani*

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ABSTRACT

The antifungal potential of chitosan obtained from shellfish was studied in both *in vitro* and *in vivo* conditions against *Pyricularia oryzae* and *Rhizoctonia solani*, causal agents of the blast and sheath blight diseases in rice, respectively. A total of 100% inhibition of mycelial growth was observed on both *P. oryzae* and *R. solani* when a 4% concentration of chitosan was used in this study. A significant reduction in both disease incidence and disease severity was observed between the treated and untreated rice plants. The disease controlling efficacy of chitosan was concentration-dependent with a negative correlation. The disease reduction (DR) capacity of chitosan in this study ranged between 47-95%. Chitosan was able to reduce disease severity (DS) of blast by 85% and sheath blight by 95% while disease incidence (DI) of blast by 77% and sheath blight by 89%. The results demonstrated that chitosan extracted from shellfish has the potential to be developed as a biopesticide for sustainable control of both blast and sheath blight diseases in rice and has broad-spectrum capacity in controlling both diseases.

Keywords: Biopesticide, chitosan, rice blast, sheath blight

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INTRODUCTION

Rice is the world's most important food crop and serves as the main source of carbohydrate for many people around the world (Dorairaj et al., 2017). Food security is jeopardized by the increasing world population. Global rice production was estimated to increase by 30% to meet the global food demand in 2030 (Wang

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et al., 2009). However, rice production is threatened by diseases caused by various pathogens. Two (2) most economically important fungal diseases of rice are blast and sheath blight (Yin et al., 2010).

Both blast and sheath blight diseases are caused by fungal pathogens, *Pyricularia oryzae* and *Rhizoctonia solani*, respectively. Depending on the environmental factors, both diseases can cause yield loss of up to 85% (Singh et al., 2015). Several fungicides have been successfully employed to control these diseases. However, the emergence of resistant fungal populations, the increasing public awareness on the negative effects caused by excessive application of fungicides and their residues on human health, and the rising demands for chemical-free food have led to a search for safer and more sustainable disease management strategies (Zahid et al., 2014). Apart from chemical control (propiconazole), several cultural practices, namely, field sanitation, crop rotation, and maintaining low seeding rate can control these diseases to a certain extent but their efficacy is inconsistent (Bag, 2009). Hence, there is a need to explore new and ecological-friendly approaches to minimize the application of chemical fungicides such as the use of chitosan as an alternative.

Chitosan [poly-(1-4)-β-D-glucosamine], also known as deacetylated chitin is a marine-based (shellfish) biopolymer comprise of high molecular weight cationic polysaccharide (Yin et al., 2010). Chitosan is found to be one of the few cationic polymers found in nature. This polycationic nature and the length of the polymer play a key role in the fungicidal property of chitosan. Chitosan which is positively charged by the protonated NH^{3+} groups interacts with the negatively charged microbial cells creating electrostatic forces that inhibit the growth of fungi (Lawrie et al., 2007) by developing internal osmotic imbalance and hydrolyzing the peptidoglycans in the cell membrane leading to the leakage of internal electrolytes such as potassium ions and low molecular weight proteinaceous constituents such as protein, nucleic acid, and glucose (Bautista-Baños et al., 2006).

Chitosan is known to induce various defense responses including the production of pathogenesis-related proteins as well as phytoalexin (Hassan & Chang, 2017). Chitosan was demonstrated as non-toxic, biodegradable, biocompatible, and possessed antimicrobial properties as well as used to produce an edible coating on fruits to increasing its shelf-life (Zahid et al., 2014). The edible nature of chitosan presents the most desirable aspect to be utilized for disease management in rice. Apart from these, nano/micro-sized chitosan has been used to protect various plants from pathogen infection such as dragon fruit, maize, bell pepper, and cucumber (Elsoud & El Kady, 2019). Liu et al. (2012) had also conducted studies to evaluate the effect of various chitosan to control sheath blight disease. In this study, we have assessed the efficacy of chitosan against both blast and sheath blight diseases in rice (*Oryza sativa*).

MATERIALS AND METHODS

Fungal Strains, Culture Conditions, and Plant Materials

Stock cultures of *P. oryzae* and *R. solani* were obtained from the Culture Collection Unit, Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia. *P. oryzae* and *R. solani* were sub-cultured and maintained on Potato Dextrose Agar (PDA) with a pH of 6, at 27±1°C, and alternating light and dark cycle.

Rice seeds of MR219 variety were obtained from the Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia. The experiment was conducted in the Laboratory of Mycology, Department of Plant Protection and greenhouse, Faculty of Agriculture, Universiti Putra Malaysia.

Preparation of Chitosan Solution

Chitosan powder from shellfish obtained from Pro Advance Technologies Sdn. Bhd. was used as a stock solution by mixing thoroughly 5 g of chitosan powder into 95 g acetic acid. The chitosan powder was made from shellfish. Chitosan solutions with concentrations of 1, 2, and 4% were prepared by diluting the stock solution with sterile distilled water.

In vitro Screening of Chitosan against *Pyricularia oryzae* and *Rhizoctonia solani*

Using Poison Agar Assay as described by Bautista-Baños et al. (2004), the preliminary screening was tested using three different chitosan concentrations and control with six replications for each treatment. PDA was poured into 90 mm diameter Petri plates. Then, 200 µL of each concentration: 1% (T1), 2% (T2), and 4% (T3) was spread over the PDA medium with a sterilized L-shaped glass rod. Control plates contained PDA added with 200 µL of acetic acid (5 mL water mixed with 95 mL acetic acid). A fungal plug of 7 mm diameter from a pure culture of 10 days old *P. oryzae* and *R. solani* were inoculated on the center of the plates, respectively. Petri plates were incubated at 28±2°C for 5 days (Bautista Baños et al., 2004).

Percent inhibition of radial growth (PIRG) was calculated according to Hayman et al. (2017):

$$\mathrm{PIRG}(\%) = [(\mathrm{C} - \mathrm{T})/\mathrm{C}] \times 100$$

where C - mycelium average growth on the control plate (cm); T - mycelium average growth on the treated plate (cm).

In vivo Screening of Chitosan against *Pyricularia oryzae* and *Rhizoctonia solani*

Seed Preparation. Rice seeds were surface sterilized with benomyl fungicide for 18 h to prevent any microbial infection. The seeds were soaked in distilled water and dried for 24 h. Germinated seeds were selected and sowed on trays until they produced true leaves (on the 14th day). On Day 15, the plants were transplanted into pots containing 5 kg soil (3: 2: 1 - topsoil: sand: compost). The water level was maintained at 1–2 cm above the soil surface during the early growth stage and was further raised to 5–7

cm at the later growth stage (Hashim et al., 2015). Each treatment was replicated five times and each replication consisted of four plants in a pot.

Chitosan Application on Rice Plants. Only the best two concentrations tested *in vitro* were selected for the greenhouse study. Leaves sprayed with 2% (T1) and 4% (T2) chitosan until run-off was performed as described by Liu et al. (2012) at 20 days after transplant with the aid of a hand-held sprayer. Control plants were sprayed with distilled water until run-off (T3).

Inoculum Preparation. *Pyricularia oryzae* inoculum was prepared as described by Tuhina-Khatun et al. (2015). *P. oryzae* was maintained on PDA and incubated in the growth chamber at 28±2°C. Conidia spores were harvested at 21 days. Spore density was adjusted to 2×10^5 spores/mL using haemacytometer and 0.05% Tween 20 was added to the spore suspension as an adjuvant before inoculation.

Rhizoctonia solani was maintained on PDA and incubated in the growth chamber at 28±2°C for five days. The mycelium was cut into plugs of 5 mm diameter using sterilized cork borer and used as inoculum (Tuhina-Khatun et al., 2015).

Inoculation of MR219 Rice with *Pyricularia oryzae* and *Rhizoctonia solani*. Both *P. oryzae* and *R. solani* were inoculated on the 21st day after planting. *P. oryzae* was inoculated by spraying 25 mL spore suspension of 2×10^5 spores/mL onto the whole plant. In the case of *R. solani*, mycelial plugs were placed on the stems at one cm below the axial of fully mature leaf and wrapped with parafilm (Khaing et al., 2015; Park et al., 2008). After pathogen inoculation, the plants were covered with plastic bags for 12 h to stimulate infection.

Experimental Design. A completely randomized design (CRD) was implemented in a pot experiment with five replications for each treatment. The same experimental design was used for both the fungi. Three (3) treatments were conducted: T1 (2% chitosan + pathogens), T2 (4% chitosan + pathogens), and T3 (0% chitosan + pathogens) as control.

Disease Assessment. Each disease was assessed on the seventh day after inoculation using a disease rating scale as shown in Table 1 (Khaing et al., 2015).

Disease incidence (DI) was calculated based on the following equation (1) (Maclean et al., 2002) as follows:

$$\text{Disease incidence (\%)} = \frac{\text{Total number of infected tillers}}{\text{Total number of tillers per hill}} \times 100\% \quad (1)$$

Disease severity (DS) was calculated based on the following equation (2) (Lim & Heong, 1984):

$$\text{Disease Severity (\%)} = \frac{\sum \text{N umber of seedlings in the rating X rating number}}{\text{Total number of seedling assessed X highest rating}} \times 100 \quad (2)$$

Disease reduction (DR) was calculated based on the following equation (3):

$$\text{Disease Reduction (\%)} = \frac{\text{DSc} - \text{DSt}}{\text{DSc}} \times 100 \quad (3)$$

where DSc - disease severity of control plants; DSt – disease severity of treated plants.

Table 1
Blast and sheath blight disease rating scale used in this study

Points	Description	
	[a]Blast disease	[b]Sheath blight disease
0	No symptoms	
1	Small brown specks of pinpoint size	Restricted dark brown oval lesions at waterline or infection points
2	Small roundish to slightly elongated, necrotic grey spots, about 1-2 mm in diameter, with a distinct brown margin. Lesions are mostly found on the lower leaves	Few oval or coalesced lesions with broad borders on lower sheaths or at infection points, 5% or less of tissue affected
3	Lesion type same as in 2, but a significant number of lesions on the upper leaves	Lesions on lower leaf sheaths or at infection points, lesions coalescing, less than 10% of tissues affected.
4	Typical susceptible blast lesions, 3 mm or longer infecting less than 4% of leaf area	Lesions mainly restricted to sheaths on the lower third of plant, lowest leaves, or other infection points, lesions discrete or coalescing with narrow red-brown border, 10 to 15% of leaf and sheath tissues affected
5	Typical susceptible blast lesions of 3 mm or longer infecting 4-10% of the leaf area	Lesions coalescing with large necrotic centers and narrow red-brown borders, 15 to 25% of tissues affected
6	Typical susceptible blast lesions of 3 mm or longer infecting 11-25% of the leaf area	Lesions extending to blades of lower leaves or lower leaves killed by injury to the sheath, 25 to 40% of tissues affected
7	Typical susceptible blast lesions of 3 mm or longer infecting 26-50% of the leaf area	Lesions extending to leaf blades of lower two-thirds of plant, 40 to 60% of tissues affected
8	Typical susceptible blast lesions of 3 mm or longer infecting 51-75% of the leaf area many leaves are dead	Lower and middle leaves dead or dying, 60 to 80% of tissues affected
9	Typical susceptible blast lesions of 3 mm or longer infecting more than 75% leaf area affected	Lesions reaching to flag leaf, lower leaves mostly dead, sheath dried, culms brown, collapsing, most tillers lodged, over 80% of tissues affected

Note. [a]Disease assessment score for blast disease (Lim & Heong, 1984); [b]Disease assessment score for Sheath blight disease (Khaing et al., 2015)

Statistical Analyses

All data were subjected to analysis of variance (ANOVA) (SAS, Cary, USA) according to the experimental design used in this study and the least significant difference (LSD) was utilized to compare

the different means of treatment. The correlation analysis was performed using Microsoft Excel 2010.

RESULTS

In vitro Screening of Chitosan against *Pyricularia oryzae* and *Rhizoctonia solani*

Mycelial growth of *P. oryzae* and *R. solani* was inhibited by chitosan of different concentrations (Figure 1) and the efficacy of chitosan to inhibit the fungal growth was concentration-dependent. For *P. oryzae*, no significant mycelial inhibition was observed in T1 (1% chitosan) with only 0.4% of PIRG. As the concentration of chitosan was increased to 2% (T2), the PIRG value increased to 26.5%. An absolute inhibition of *P. oryzae* mycelial growth was observed on plates with 4% chitosan (T3).

A similar trend was observed in the inhibition of *R. solani*. Chitosan of 1% (T1) inhibited slightly the mycelial growth of *R. solani* with PIRG 3.5% while chitosan of 2% (T2) was able to increase PIRG up to 30% and chitosan of 4% (T3) had achieved total inhibition of mycelial growth with PIRG 100%.

In vivo Screening of Chitosan against *Pyricularia oryzae* and *Rhizoctonia solani*

Disease Incidence and Disease Severity. Disease incidence (DI) and disease severity (DS) of rice plants infected with *P. oryzae* and *R. solani* in separate trials were tabulated in Table 2. Disease development in rice plants occurred between 7 to 12 days after the inoculation of both fungi separately. For *P. oryzae*, a pinpoint-sized brown color speck appeared on the seventh day and developed into diamond-shaped lesions with brown borders (Figure 2a) in five plants infected with *P. oryzae* in the control treatment (T3, 0% chitosan). There was a significant ($p \leq 0.005$) difference in both DI and DS between the treated (T1-2% chitosan, T2-

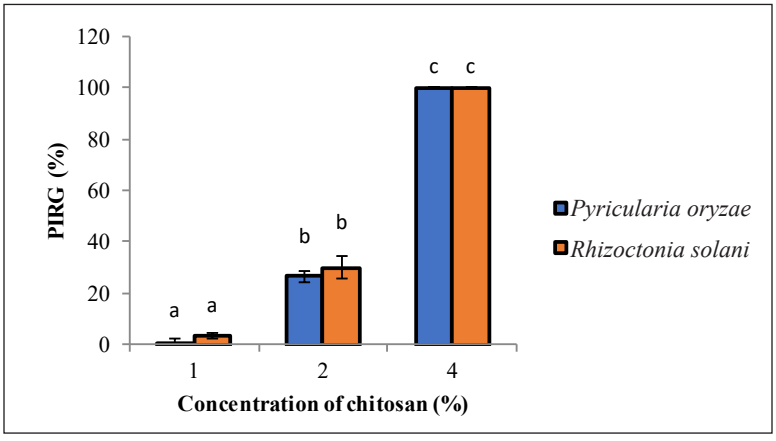

Figure 1. Percent inhibition of radial growth (PIRG) of *Pyricularia oryzae* and *Rhizoctonia solani* at 5 days after plating using poison agar assay with different concentrations of chitosan on PDA medium. Vertical bars indicate standard error of six replicates. Bars with the same alphabets are not significantly different. The standard error for concentration 4% was zero

Table 2
Disease incidence and disease severity for blast and sheath blight diseases on rice plants with or without treatment of chitosan at 7 days after pathogen inoculation

	Blast disease			Sheath blight disease		
Treatment	T1	T2	T3	T1	T2	T3
Disease Incidence (%)	44.7±1.6^{b}	19.4±1.5^{c}	84.1±5.3^{a}	28.8±5.5^{b}	6.1±5.6^{c}	56.4±7.1^{a}
Disease Severity (%)	22.8±1.6^{b}	10.9±0.6^{c}	71.1±5.8^{a}	7.8±1.7^{b}	1.0±0.8^{b}	22.9±9.9^{a}

Note. T1 = 2% chitosan; T2 = 4% chitosan; T3 = 0% chitosan. Values are mean of five replications. Values with the same alphabets in the same row for a respective disease are not significantly different

(a) (b)

Figure 2. Symptoms of rice blast (a) and sheath blight (b) diseases at 12 days after inoculation

4% chitosan) and the control (T3) plants. The control plants (T3) exhibited two folds higher DI (84.1%) and DS (71.1%) compared to the 2% chitosan treated (T1) plants (44.7% and 22.8%, respectively). The lowest DI (19.4%) and DS (10.9%) were observed in plants treated with 4% chitosan (T2).

A similar trend was observed for sheath blight disease. However, the intensity of sheath blight disease was slightly lower than the blast disease. The appearance of a very small dark brown lesion that was oval-shaped was observed in the control (T3, 0% chitosan) plants on the seventh day and developed into irregular lesions with white-gray centers and brown margins (Figure 2b). The result also revealed a significant difference in the values of DI and DS between the treated (T1, T2) and control (T3) plants. Rice plants treated with 4% chitosan (T2) were shown to be effective in controlling sheath blight disease with the minimum DI (7.8%) and DS (1.0%). However, no significant difference was observed in DI and DS between plants treated with 2% (T1) and 4% (T2) chitosan.

Disease Reduction (DR). Chitosan was shown to be effective in controlling both blast and sheath blight diseases in MR219 plants. For disease incidence (DI), there was no significant difference in DR of plants treated with 2% chitosan between blast and sheath blight diseases (46.8 and 48.9%, respectively) (Figure 3). The highest DR was observed in plants pre-treated with 4% chitosan and challenged with *R. solani* (89.1%) followed by those challenged with *P. oryzae* (76.9%) and there was also no significant difference in DR between the two diseases. However, there was a significant difference in DR between the concentrations used (2% vs 4%) for both blast and sheath blight diseases. Chitosan at 4% demonstrated significantly higher DR for DI compared to chitosan at 2% in both diseases.

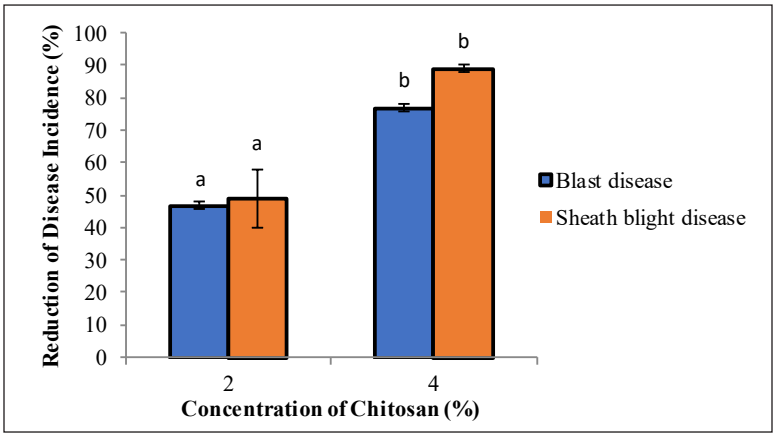

Figure 3. Disease reduction (DR) in disease incidence (DI) of the blast and sheath blight diseases in greenhouse condition with two different concentrations of chitosan as treatments on rice plants. Vertical bars indicate standard error of five replicates. Bars with the same alphabets are not significantly different

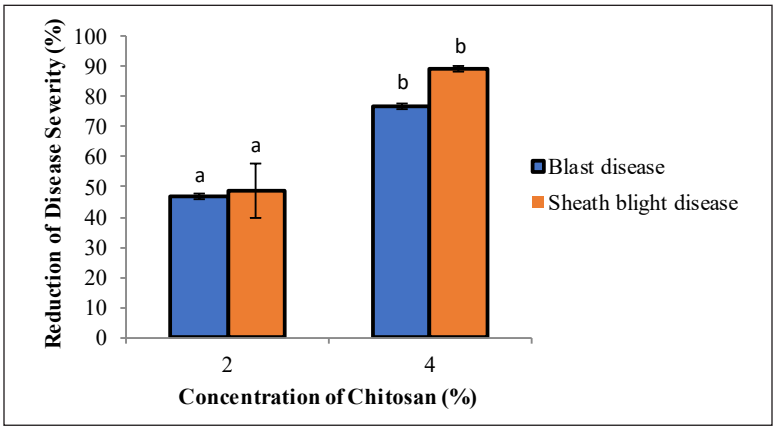

Figure 4. Disease reduction (DR) in disease severity (DS) of the blast and sheath blight diseases in greenhouse condition with two different concentrations of chitosan as treatments on rice plants. Vertical bars indicate standard error of five replicates. Bars with the same alphabets are not significantly different

A similar trend was observed for disease severity (DS) (Figure 4). At 2%, there was no significant difference in DR between plants challenged with *P. oryzae* (67%) and *R. solani* (61%), and at 4%, plants challenged with *P. oryzae* (84.5%) and *R. solani* (95.0%). However, for both diseases, there was a significant difference in DR between the concentrations used (2% vs 4%). Chitosan at 4% demonstrated significantly higher DR for DS compared to chitosan at 2% in both diseases.

Correlation Analysis

Table 3 shows the correlation analysis (*r* values). For blast disease, the *r*-value (-0.6) between the treatment and DI was weak and negatively correlated but the *r*-value (-0.9)

Table 3
Correlation analysis between the treatment, disease incidence and disease severity in rice plants

Blast disease			
	Treatments	Disease Incidence	Disease severity
Treatments	**1**		
Disease incidence	-0.60	**1**	
Disease severity	-0.94	0.83	**1**
Sheath blight disease			
Treatments	**1**		
Disease incidence	-0.99	**1**	
Disease severity	-0.98	0.99	**1**

between the treatment and DS was strong and negatively correlated. The negative *r* value indicates that the higher chitosan concentration used a higher reduction in DI and DS values in rice plants. This indicates that chitosan demonstrated a stronger effect in the reduction of DS than DI. In the case of sheath blight disease, the interaction between treatment, DI and DS was strong and negatively correlated (r = -0.9). In conclusion, chitosan has the potential to control blast and sheath blight diseases in the field.

DISCUSSION

The positive effects of chitosan have been documented in various pathosystems involved in a wide range of plants including both monocotyledon (rice) and dicotyledon (bell pepper, cucumber, dragon fruit), and a diverse range of pathogens including fungi, bacteria, and viruses (Zahid et al., 2014). In this study, we studied the effects of chitosan on rice against the infection of two important fungal pathogens, namely *P. oryzae* and *R. solani*.

In this study, the *in vitro* antifungal test revealed that chitosan was effective against both *P. oryzae* and *R. solani*, which inhibited the mycelial growth of both fungi at the highest concentration used (4%). The rate of inhibition of radial growth by chitosan was concentration-dependent similar to the biostimulants (Surendran et al., 2017). Other studies reported a similar effect when chitosan was used against various plant pathogenic fungi *in vitro*. When the concentration of chitosan was increased from 0.75 to 6.0 mg mL^{-1} in the PDA medium, decrement in the radial growth of *Alternaria alternata*, *Botrytis cinerea*, *Rhizopus stolonifer*, and *Colletrotichum gloeosporioides* was observed (El Ghaouth et al., 1992). A similar effect was also reported in *Sclerotinia sclerotiorum* when chitosan concentration was increased from 1 to 4% (W/V) (Junior et al., 2016). Complete inhibition of the fungi *R. stolonifer*, *Fusarium oxysporum*, *Penicillium digitatum*, and *C. gloeosporioides* was obtained at a concentration of 3% (w/v) (Bautista-Baños et al., 2003, 2004).

Similarly, in this study, the *in vivo* trial results demonstrated that chitosan at 4% concentration was effective to control both blast and sheath blight diseases with more than 80% disease reduction. Chitosan was used to control various *Fusarium* spp. in various economically important hosts including *Fusarium oxysporum, Fusarium graminearum*, and *Fusarium solani* in tomato, wheat, and peas, respectively by reducing DI more than 50% (Al-Hetar et al., 2011; Prapagdee et al., 2007; Sharp, 2013). It was also found that chitosan (0.2 mg/mL) had induced a delayed disease appearance in rice plants (three weeks old) and thus, reduced the disease symptoms in plants (Liu et al., 2012). Boonreung and Boonlertnirun (2013) reported that chitosan sprayed at a concentration of 40 mg/L for four times throughout the crop season before the inoculation of *Helminthosporium oryzae*, *Curvularia lunata*, and *Fusarium moniliformae* reduced 12% of dirty panicle diseases in rice. However, in this study, 4% chitosan spray once throughout the crop season before inoculation of *P. oryzae* and *R. solani* was able to reduce disease severity by 85 and 95%, respectively.

Apart from disturbing the cell wall of the pathogens, chitosan by itself can be a physical barrier to pathogen attack by creating a barrier film or chelating the minerals and make them inaccessible to the pathogens. Chitosan is capable of eliminating the necrotrophic pathogens by neutralizing the mycotoxin produced by these pathogens (Sudarshan et al., 1992). Hence, we speculate that the inhibitory effect of chitosan against *P. oryzae* (a hemibiotroph) in this study was by creating a barrier film or chelating minerals and against *R. solani* (a necrotroph) by neutralizing the mycotoxins produced. These phenomena indicate that chitosan may use a double mechanism of actions to control both types of pathogens.

The nano-sized chitosan was able to control blast disease caused by *Pyricularia grisea* with 100% disease reduction by inducing systemic acquired resistance (SAR) in rice (Xing et al., 2015). Most of the chitosan used in the above-mentioned studies were modified into different forms including nano-sized chitosan because some evidence stated that the negative effect of chitosan on plant growth, shoot length when used in higher concentration in the natural form has been reported (Sandford, 2003). However, the natural chitosan obtained from shellfish used in our study at 4% had revealed a superior disease reduction capacity of 95.0% against sheath blight and 84.5% against blast disease. Chitosan has been produced from various sources ranging from fungi to plants. However, chitosan extracted from shellfish will incur lower costs because it is produced from waste products of the seafood industry (Peniche et al., 2008).

Chitosan and its derivatives have emerged as the best eco-friendly bio-pesticides in the last few decades (Peniche et al., 2008). Other than controlling various plant diseases, chitosan also increases the population of nitrogen-fixing bacteria and vesicular-arbuscular mycorrhizal fungi

(VAM) (Bautista-Baños et al., 2006). US Environmental Protection Agency in 2015 has concluded that chitosan produces no negative impact on the environment (Hassan & Chang, 2017). Due to its eco-friendly and low production cost, chitosan has a huge potential to be used as a biocontrol strategy for sustainable disease management, not only in rice but in other crops as well.

CONCLUSION

To the best knowledge of the authors, this is the first report on the high efficacy and broad-spectrum capacity of chitosan at low concentrations for the control of both blast and sheath blight diseases of rice. Chitosan at 4% concentration had shown a disease severity reduction capacity of 85 and 95% in blast and sheath blight diseases, respectively. To validate this result, field study is required to test the consistency of chitosan in controlling these two fungal diseases in rice. Due to the high demand of rice around the world, these findings could potentially bridge the yield gap in the near future as well as contribute to a sustainable crop production system.

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