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Research Article

The Role of Nanochitosan on the Expression of Rice Resistance Genes against Bacterial Leaf Blight

Siswanti[1)], Tri Joko[2)], & Siti Subandiyah[2)]*

[1)]Study Program of Biotechnology, The Graduate School of Universitas Gadjah Mada, Jln. Teknika Utara, Pogung, Mlati, Sleman, Yogyakarta, 55281 Indonesia

[2)]Department of Plant Protection, Faculty of Agriculture, Universitas Gadjah Mada Jln. Flora No. 1, Bulaksumur, Sleman, Yogyakarta 55281 Indonesia

**Corresponding author. E-mail: sitisubandiyah@.ugm.ac.id*

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ABSTRACT

Bacterial leaf blight (BLB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) has been reported to cause ± 20–50% of rice yield loss around the world. Resistant varieties are used to control this disease, however due to rapid evolution of this pathogen, the resistances was broken down in a few years. This study is aimed to determine the role of nanochitosan in the expression of rice *Xa21* and *Xa1* resistant genes against *Xoo*. The BLB susceptible rice cultivar IR64, the *Xoo* isolate MAG2 and a 0.065% concentration of nanochitosan were used in this experiment. Application of nanochitosan was carried out within 1-week intervals starting at rice aged 2–10 weeks after transplanting. The expression of *Xa21* and *Xa1* genes against Xoo were analyzed using conventional PCR and qPCR methods at 0 and 4 days after *Xoo* inoculation followed by 4x scoring of disease symptoms in 1-week interval. The treatments used in this study included the mock one/inoculated with sterile distilled water, K (+)/ plants inoculated with Xoo, CNP (-)/ with nanochitosan and sterile distilled water inoculation, and CNP (+)/ with nanochitosan and *Xoo* inoculation. The results showed that the 0.0065% concentration nanochitosan application was able to increase the expression of *Xa21* and *Xa1* genes on CNP (-). Disease intensity and AUDPC values did not show any significant difference between K (+) and CNP (+). This study concluded that nanochitosan at 0.065% was able to increase the expression of rice *Xa21* and *Xa1* resistance genes. However, the gene expression was not able to significantly suppress the infection development of *Xoo*.

Keywords: Bacterial Leaf Blight; nanochitosan; rice

INTRODUCTION

Bacterial leaf blight (BLB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) on rice, is an important disease that has been reported to reduce production globally (Adhikari *et al*., 1995). Yield loss due to this disease is estimated to reach 20-50% on severely infected fields and 10–20% if infection occurs at maximum vegetative stages (Wang *et al*., 2005).

The most used management practice for *Xoo* is to use resistance varieties; however, the high evolution rate of this pathogen has caused this pathogen to overcome plant resistant characters (Suryadi *et al*., 2011; Joko *et al*., 2019). Combining various management technique may serve as a solution to manage the evolution issue to this pathogen. Chitosan has been recently developed as a plant elicitor, which is considered to be economically effective and able to induce rice resistances against BLB (Modina *et al*., 2009).

Chitosan may act as a pathogen/microbe-associated molecular pattern (PAMP/MAMP) in various pathosystems. PAMP/MAMP may be effectors that is secreted by pathogens. Chitosan will be recognized by plant pattern recognition receptor (PRR) and cause resistant responses from plants (Hadrami *et al*., 2010). Chitosan in large amounts are difficultly dissolves in liquid solvents setting a challenge when applied on fields. A solution to this challenge, is by formulating chitosan into nanoparticles. Nanoparticle-sized chitosan (CNP) has been used in agriculture due to its biodegradability, solubility, high permeability, non-toxic effects on humans, low prices, and effectivity compared to larger-sized chitosan (Manikandan & Sathiyabama, 2015). This study was conducted to determine the effect of CNP solution on the expression of the resistant genes, *Xa21* and *Xa1,* on rice variety IR64, which is susceptible against *Xoo*.

MATERIALS AND METHODS

Xanthomonas oryzae pv. oryzae Culture Preparation

The *X. oryzae* pv. *oryzae* strain used was obtain from the culture collection of Research Center for Biotechnology, Universitas Gadjah Mada, Yogyakarta. The *Xoo* MAG 2 was isolated from infected rice, Ciherang variety, collected from Magelang, Central Java. Isolates were grown on solid peptone sucrosa agar (PSA) and incubated at 28°C for 3 days in order to activate *Xoo* (Joko *et al*., 2000). Solid PSA medium (pH 7.0) per liter contained 5 g of peptone; 0.5 g of K_2PO_4; 0.25 g of $MgSO_4.7H_2O$, and 20 g of sucrose.

Rice Plants and Nanoparticle-sized Chitosan Application

This study was done from February to November 2018 at Reseach Center for Biotechnology, Universitas Gadjah Mada, Yogyakarta. Rice seeds, variety IR64 and labelled as stock seeds, were obtained from Yogyakarta Assessment Institute for Agricultural Technology (BPTP) Yogyakarta. The experiment consisted of 4 treatments: 1) mock: plants were inoculated with sterile distilled water and without CNP applications; 2) a positive control/C(+): plants were inoculated using *Xoo* and without CNP applications; 3) without CNP application/CNP(-): plants were inoculated with *Xoo* and without CNP; 4) with CNP/CNP(+): plants were inoculated with *Xoo* and applied with CNP. Each treatment was replicated 4 times with each pot containing 2 rice plants. As much as 30 mL of CNP were applied according to each treatment (Modina *et al*., 2009). CNP solvents were made using an ionic gelation method (Handani *et al.*, 2017) at concentration of 0.065%, pH 3.31 and particle size of 150.2 nm based on the Particle Size Analyzer (PSA). This is caused due solvents agglomerated after being left for a couple of hours at concentration of < 0.06%. Therefore, solubility was not stable based on this experiment. CNP were applied weekly starting from rice seedlings 2 to 10 weeks after transplanted from seedbeds and grown for 2 weeks. The negative and positive control were applied with sterile distilled water with similar volumes and application intervals.

Xanthomonas oryzae pv. oryzae Inoculation on Rice Plants

Inoculums of *Xoo* were subcultured on liquid peptone sucrose medium and incubated at 28°C for 2 days. The suspensions of *Xoo* turbidity or density were measured before inoculation using a spectrophotometer at OD_{600} = 0.5 or equivalent to colony density of ± 242 × 10^6 cfu/ml. Inoculation of BLB causing pathogens were done using the leaf clipping method (Yinggen *et al*., 2017) when rice were 41 days old. Similar clipping method were done on mock and K (+) with sterile distilled water.

Analyzing Expression of Resistant Genes, Xa21 *and* Xa1, *of Rice*

Total RNA was extracted from leaves with and without CNP treatments at 0 days and 4 days after *Xoo* inoculation. RNA was extracted from plant tissue using *Rneasy Plant Mini Kit* (Qiagen). Isolated RNA was then synthesized into cDNA using cDNA kits. The resistant genes *Xa21* and *Xa1* were first confirmed using conventional PCR reaction to determine the existence of both genes and optimum annealing temperature for real time PCR. Annealing temperature and time for *Xa21*, *Xa1*, and ubiquitin, a gene used for internal kontrol, respectively were 61°C for 45 s, 59°C for 45 s, and 55°C for 30 s (Sutrisno *et al.*, 2018). Real Time PCR (Bio-Rad CFX96) were done according to protocols and replicated twice for each sample. Threshold cycle (Ct) values from results of targeted genes form real time PCR were normalized with ubiquitin *Oryza sativa* Ct value and analyzed using a livak method (Livak & Schmittgen, 2001). Value 2^-ddct from the calculations show the folds of change of gene expressions. Primers used for real time PCR (Table 1) specificity were checked through *https://www.ncbi.nlm.nih.gov/tools/primer-blast/primertool.cgi*.

Bacterial Leaf Blight Symptoms Observation

Observation of BLB symptoms caused by *Xoo* were defined in percentages of disease intensity (Strange, 2003) and Area Under the Disease Progress Curve (AUDPC) were calculated using formulas as described by Ahmed *et al.* (1999). Morphological symptoms from leaf blight lesions were observed according to Rusli *et al*. (2016). Scale of BLB infection were determined based on Standard Evaluation System (SES) (IRRI, 2013). Percentages of leaf area infected were determined as disease incidences and calculated as described by Wheeder (1969). BLB symptoms were measured at 1, 2, 3, and 4 weeks after *Xoo* inoculation. Observation data were analyzed using Mann-Whitney SPSS to determine significant differences at α=0.05.

Table 1. Specific primer for gene *Xa21*, *Xa1*, and *ubiquitin*

Gene		Sequences (5' -> 3')	Base number	Tm(ºC)	Product length (bp)
Xa21	Forward	CTCGGCGACAACTACCTCTC	20	60.01	151
	Reverse	GGCTGAGGTCTAGCGATGTC	20	59.98	
Xa1	Forward	ATGTGGGGCAAAACTGAAAG	20	59.97	289
	Reverse	TTCAAGACCTTCGAGCACCT	20	59.99	
ubiquitin	Forward	CACAAGAAGGTGAAGCTCGC	20	56.4	181
	Reverse	CCTTCTGGTTGTAGACGTAGG	21	54.5	

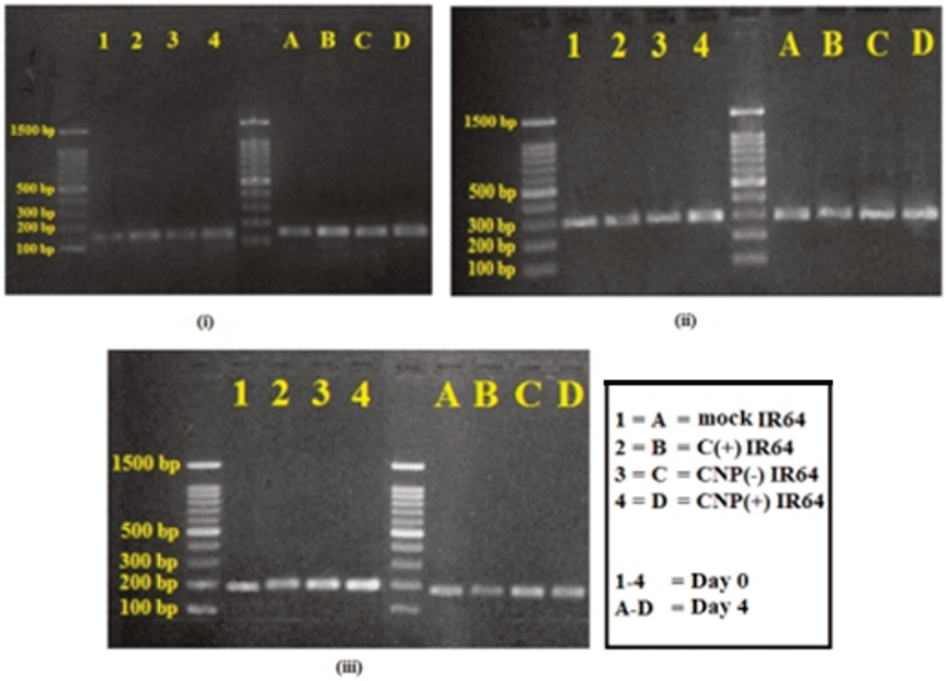

Figure 1. Electrophoresis *Xa21* size 151 bp (i), *Xa1* size 289 bp (ii), and ubiquitin size 181 bp (iii) on rice, variety IR64; mock = negative control inoculated with sterile distilled water, C = control, CNP = Chitosan Nanoparticles, (-) = inoculated with sterile distilled water, (+) = inoculated with *Xanthomonas oryzae* pv. *oryzae*

RESULTS AND DISCUSSION

All cDNA samples from all treatment tested reverse transcriptase RNA were confirmed to contain the expression of *Xa21* (Figure 1). Expression of *Xa21* from all treatments indicated that the expressions of this gene is related to either inoculation using sterile distilled water, *Xoo,* or CNP application. Leaves that were not treated with *Xoo* still expressed *Xa21;* therefore, *Xa21* is constitutively expressed by leaf tissue. The gene *Xa21* is expressed from resistant and susceptible variety, but not determine by the infection of *Xoo* or mechanical damages (Century *et al*., 1999).

The *Xa1* gene was also expressed from all cDNA reverse transcriptase RNA samples from all treatments (Figure 1). Different from *Xa21*, *Xa1* expression is induced from *Xoo* inoculation or mechanical damages (Yoshimura *et al*., 1998). The expression of *Xa1* from the mock and CNP(-) is induced due to mechanical damage from the clipping method.

Results of disease intensity showed that intensities were lower on plants treated with CNP compared to the C(+) only on week 4 (Figure 2), implying that application using CNP 8 times (4 times after inoculated with *Xoo*) with an 7 day interval was sufficient to decrease BLB intensity. This may be caused by the low concentration of CNP or time intervals between application were too long after inoculation with *Xoo*. The *Xa21* gene is fully expressed at tillering (Park *et al*., 2011). Therefore, it would be more beneficial if CNP applications to induce resistant genes were done after rice plants reach maximum tillering. The AUDPC value from the positive control was not

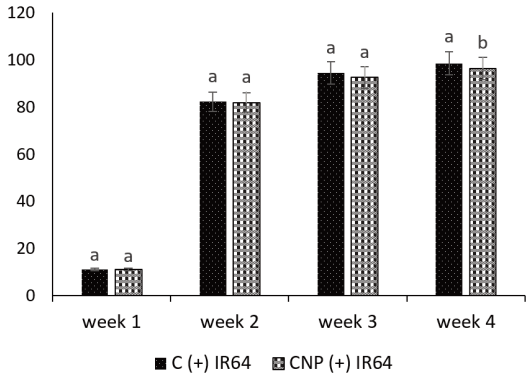

Figure 2. Average percentage of disease intensity from week 1–4 and AUDPC on rice variety IR64; C = Control, CNP = Chitosan Nanoparticles, (+) = inoculated using *Xanthomonas oryzae* pv. *oryzae*; different letters indicated significant differences at α=5% (Mann-Whitney test)

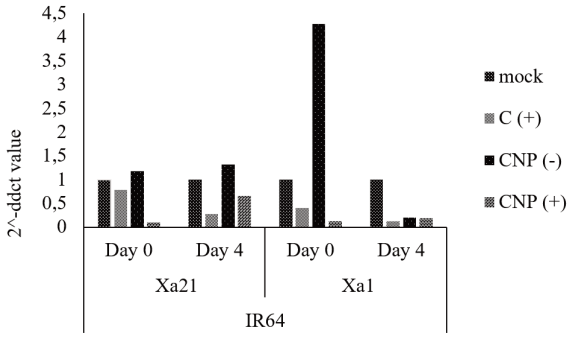

Figure 3. The 2^ddct value of gene *Xa21* and *Xa1* from rice variety IR64 at 0 and 4 days; *mock* = control inoculated with sterile distilled water, C = Control and CNP = Chitosan Nanoparticles, (-) = inoculated with sterile distilled water, (+) = inoculated with *Xanthomonas oryzae* pv. *oryzae*

significantly different from plant treated with CNP (Figure 2), causing solvent not being able to prevent BLB development.

The 2^-ddct value for variety IR64 showed an increase of *Xa21* resistant gene after CNP application for CNP (-) for 1.18-folds at day 0 and 1.31-folds at 4 days compared to the mock. The *Xa21* gene experiences increasement that were higher on day 4 for CNP (-) compared to day 0 (Figure 3). This showed that CNP application was able to increase the expression of *Xa21* on rice variety IR64, however without *Xoo* inoculation.

When inoculated with *Xoo,* the expression of *Xa21* was lower based on its 2^-ddct being < 1, however 2^-ddct value of CNP(+) on day 4 was larger than day 0, whereas smaller on C (+) (Figure 3). Rice varietas IR64 is a susceptible variety to BLB (Wahab *et al.*, 2017), which explain the decrease of *Xa21* gene expression on day 4 after inoculated with *Xoo*, however the decrease was lower when applied with CNP. This showed that CNP was able to decrease the downregulation of *Xa21* gene expression on rice variety IR64 inoculated with *Xoo*.

Xa21 expression from the C(+) treatment at day 4 was smaller compared to day 0 (Figure 3) and may be caused by the low content of XA21 binding protein 3 *(*XB3) and high content of XB15 in plants. Protein XB3 contains an Ring Finger (RF) that interacts with the XA21 kinase domain. XB3 is specifically transphosphorylated by XA21 kinase domain. Reduction of XB3, triggers a decrease of XA21 protein and plants resistances related to XA21, therefore positively act in plant immunity mediated by XA21. Accumulation of XA21 protein complex requires XB3, however XB3 does not require XA21 for stability. If silencing of XB3 occurs, concentration of XA21 protein will decrease; however, XB3 protein accumulated in plants were similar whether *Xa21* gene were present or not (Wang *et al*., 2006).

XA21 binding protein 15 (XB15) is Protein Phosphatase 2C (PP2C), a group of serine/threonine phosphatase which acts as a monomer that requires Mn^{2+} and/or Mg^{2+} to regulate negative immunity. Dephosphorylate kinase by phosphatase protein is a common mechanism to decrease signaling through kinase. Over-expression of XB15 decreases XA21 mediated resistances against *Xoo*. XB15 is related to serin on JM (*JuxtaMembrane*) XA21 and synthesis of XA21/XB15 complex induced by *Xoo* detected 12 hours after inoculation and increase significantly after 24 hours (Park *et al*., 2008).

Based on the research done by Akamatsu *et al.* (2016), the concentration of CNP used in this study was enough to induce expressions of genes related to resistance of rice plant cells. The same research also stated that chitosan concentration > 15 µg/mL was able to induce the production of reactive oxygen spesies (ROS) and other genes related to rice resistance. Therefore, the development of BLB may be caused by other factor, such as pH. Nanoparticle-size chitosan pH solvent reached acid, specifically 3.31. This acid condition may affect

Mg^{2+} uptake by rice plants. Ion uptake is affected by pH of external solvent. The level of Mg^{2+} uptake may double at pH value of 4.5 compared at pH 6.5 after 15 minutes observations (Kobayashi & Tanoi, 2015). This causes acid condition to increase Mg^{2+} uptake by rice plants. Over-expression of XB15 decreases resistances mediated by XA21 against *Xoo* (Park *et al*., 2008). Therefore, external solvent which are acid may supply Mg^{2+} for XB15 activation. The application of CNP that were not able to increase *Xa21* expression of plants inoculated with *Xoo* may be due to the effector Xoo2875, effector type III of *Xoo* which may help suppress rice resistance. Yamaguchi *et al*. (2013) reported that disease lesions were not able to develop on wild-type rice inoculated with the mutant *Xoo* hrpX deficiency-T3SS, a mutant that disallow sending of effector type III. This mutant is able to strongly induce Pattern Triggered Immunity (PTI) on rice. On the other hand, transgenic rice that expressed Xoo2875 (Xoo2875-OX) demonstrated severe lesions. Population of mutant *Xoo* hrpX on plant Xoo2875-OX were also 100-folds compared to wild-types (Yamaguchi *et al*., 2013). Besides Xoo2875, effector type III that are able to suppress plant resistance are for example AvrPto (Xiang *et al*., 2008) and AvrPtoB (Wang *et al*., 2019) on *Pseudomonas syringae*.

The expression of Xoo2875 suppressed Brassinosteroid (BR) and MAMP response of plant inoculated by the mutant *Xoo* hrpX. Xoo2875 hinders resistance against *Xoo* dan BR response by downgrading the function of OsBAK1. Besides interacting with OsBAK1, Xoo2875 also interact with OsBiSERK1, which is close to OsBAK1 and involved in immune responses. However, Xoo2875 does not interact with OsBRI1 or Xa21. BAK1 is a common component of many MAMP receptor complex; thus, suppression the BAK1 functions is an effective strategy of PRR due to Xoo2875 blocking MAMP signaling pathway (Yamaguchi *et al*., 2013).

Long *et al*. (2018) reported that combination of 3 *Xanthomonas* outer protein (Xop), including XopN, XopV, and XoZ as a type III non-TALE (transcription activator–like effectors) effector of *Xoo,* was able to repress mitogen-activated protein kinase (MAPK) activity and has a role in virulence when *Xoo* infect and forms lesion symptoms. MAPK is a PTI response due to peptidoglikan, a common PAMP molecule on *Xoo*.

The 2^-ddct value showed an increase of *Xa1* expression on plants treated with CNP and inoculated with sterile distilled water by 4.27-folds compared to the negative control at day 0 (Figure 3).

Expression of *Xa1* decreased on rice plant treated with CNP and *Xoo* and measurements from day 4 were slightly larger than day 0. However, 2^-ddct values from C(+) on day 4 were smaller than day 0 (Figure 3). This implies that CNP can decrease the downregulation of *Xa1* expression of rice variety IR64 after inoculated with *Xoo* even though only slightly. The gene *Xa1* will recognize whole TALE of *Xoo* as an avirulen effector. Previous TALE variants are named pseudogene, also named interfering TALE (iTALE). iTALE is a mutant TALE on their stop codon premature or large deletions on the 3' end for code sequence, but expressed as a sliced protein on the C end. *Xoo* mutates a couple of TALE to not only avoid protein recognition of Resistance NLR, but also actively push resistance through progenitor TALE on compatible interaction (Sasaki & Ashikari, 2018).

Rice variety IR64, a susceptible variety to BLB, minorly expressed *Xa1* gene after inoculated with *Xoo* in both CNP and non–CNP treated plants, due to *Xoo* ability to avoid whole TALE recognition from *Xa1* through clipped iTALE/TALE; thus, suppressing activity of the resistant gene *Xa1*. This caused compatible interaction and CNP solvent in this study to not yet be able to suppress the effect of iTALE from *Xoo*.

CONCLUSION

Nanoparticle–sized chitosan were able to increase the expression of the resistant genes *Xa21* and *Xa1* on rice. However, expression increase was suppressed by *Xoo.* Studies regarding other genes that affect rice immunity against *Xoo* and virulent gene of *Xoo* are required. This will help the effectivity of CNP solvents in more detail as a base to development of chitosan nanoparticle in expressing resistant genes against BLB.

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