INTRODUCTION

Bacterial leaf blight (BLB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) on rice, is an important disease that 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

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 (Hadrani *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 & Sathiabrama, 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*.

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*.

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INTRODUCTION

Bacterial leaf blight (BLB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) on rice, is an important disease that has been reported to cause reduction 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 (Hadrani *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 & Sathiabrama, 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 obtained 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 sucrose 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 K₂PO₄, 0.25 g of MgSO₄·7H₂O, and 20 g of sucrose.

Rice Plants and Nanoparticle-sized Chitosan Application

This study was done from February to November 2018 at Research 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 with *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 OD₆₀₀ = 0.5 or equivalent to colony density of ± 242 × 10⁸ 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 (Surisno 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^-ΔΔCt 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](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.
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
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; different letters indicated significant differences at α=5% (Mann-Whitney test).

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 Mn2+ and/or Mg2+ 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 species (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.

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.

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 OsBRII 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).

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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