Salicylic acid-induced accumulation of biochemical components associated with resistance against *Xanthomonas oryzae* pv. *oryzae* in rice

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Introduction

Rice (*Oryza sativa* L.) is one of the most important crops in the world. Asia has the largest growing area, with top producing countries including China, India, Thailand, and Vietnam (Xu et al. 2013). Since the 1960s, with the widespread cultivation of high-yielding and nitrogen-responsive dwarf hybrid varieties, rice diseases have become more prevalent (Khush 1995). Among these diseases, bacterial leaf blight (BLB) disease caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) accounts for around 20% of annual yield loss worldwide (Shimono et al. 2012; Zhu et al. 2013). Exogenous salicylic acid (SA) can trigger the SA-dependent signaling pathway leading to the disease resistance in rice. The SA-dependent pathway is associated with systemic acquired resistance (SAR) activated upon infection of the rice plant pathogens (Sticher et al. 1997).

An important characteristic of induced resistance is the phenomenon of priming in which rice plants exhibit a more rapid and elevated expression of defence responses upon bacterial pathogen infection compared to untreated rice plants (Conrath et al. 2006). In rice plants, exogenously supplied SA is converted to POD-glucosyl-SA by the enzyme glucosyltransferase (Silverman et al. 1995), which further stimulates the production of SA. This phytohormone can directly affect pathogens as well as contribute to the establishment of SAR (Tamaoki et al. 2013). As part of induced resistance, systemic signals such as SA influence the pathogen’s virulence machinery. Previous studies have shown that exogenous SA can block biosynthesis of flagella in *Escherichia coli* (Kunin et al. 1995) and reduce virulence of *Agrobacterium tumefaciens* (Yuan et al. 2007).

SAR stimulates the expression of resistance genes. These genes can create phytoalexins, reactive oxygen intermediates, hypersensitive responses (HRs), cell wall fortification, defence enzymes, and other defence gene products that kill or inhibit pathogen growth (Thulke & Conrath 1998; Metraux 2002; Metwally et al. 2003; Shadle et al. 2003; Galis et al. 2004). Production of the glucosyltransferase enzyme has been shown to be maximally induced by 1 mM SA, with 7-fold increase of the enzyme activity at 6 h in both roots and shoots after treatment of rice with SA (Silverman et al. 1995). In another study, activity of the H2O2-degrading enzyme peroxidase decreased, while superoxide dismutase activity did not change after

FTIR transform infrared spectroscopy analysis revealed that the higher ratios of 1233/1517, 1467/1517, and 1735/1517 cm⁻¹ observed in treated rice suggested alteration of monomer composition of lignin and pectin in the rice cell wall. Exogenous SA-treated rice had more amide I β-sheet structure and lipids as shown by the peaks at 1629, 2851, and 1735 cm⁻¹. These biochemical changes of rice treated with SA and inoculated with *Xoo* were related to primed resistance of the rice plants to BLB disease.
treating leaves by floating in 2 or 5 mM SA (Ganesan & Thomas 2001). However, an increased activity of peroxidase was recorded after a foliar spray of 8 mM SA in rice to induce resistance to rice blast (Daw et al. 2008). These authors also observed enhanced polyphenol oxidase activity and production of four rice phytoalexins including three oryzalexins and momilactone A following the SA treatment. Foliar spray with SA induced the production of 36 proteins with different functions including signal transduction, antioxidant activity, and defence (Li Y et al. 2012). The induced resistance of rice against Xoo by priming with exogenous SA has not yet been characterized. Therefore, the objective of the study was to characterize the defence responses of rice plants against BLB after treatment with exogenous SA and challenge inoculation with Xoo by monitoring biochemical changes associated with plant defence mechanisms.

Materials and methods

**Resistance inducer and rice cultivar**

The rice (O. sativa) seeds of the susceptible cultivar, Khao Dawk Mali 105 (KDMML105), as well as the SA (Acros Organics, ThermoFisher Scientific, USA) used in this study were provided by the Plant Pathology and Biopesticide Laboratory (PPBL), Suranaree University of Technology, Thailand. Prior to this research, different kinds of resistance inducers at various concentrations had been screened to evaluate their effects on seed germination and BLB disease severity (DS) by the procedure of Anwar et al. (2013). Subsequently, SA at 1 mM concentration was chosen because it gave the highest efficacy in controlling the BLB disease.

**Culture conditions of Xoo**

The Xoo strain SUT1–121 (virulent strain) was provided by the PPBL, Thailand. It was retrieved by streaking the stock culture onto nutrient glucose agar (NGA) and incubated at 28 ± 2°C for 48 h. After that, the bacterial culture was propagated in 500 ml of nutrient broth containing 2% glucose (NGB) for 48 h at 28 ± 2°C with constant shaking at 180 rpm. After the incubation, the culture was re-suspended in sterile distilled water. Finally, density of the bacterial suspension was adjusted to 1 × 10^8 cfu ml⁻¹ based on its specific absorbance at 600 nm (Buensanteai et al. 2008; Nagendran et al. 2013).

**Plant cultivation, induction treatment, and Xoo inoculation**

The experiment was conducted in completely randomized design (CRD) with five replications. For the exogenous application of SA, the rice seeds (cv. KDMML105) were sterilized on the surface with 95% ethanol (v/v) for 2 min, followed by five washes with sterile distilled water to remove the alcohol residue, and soaked in water overnight. Subsequently, 10 g of the treated rice seeds were soaked in 50 ml of 1 mM SA solution, germinated on wet filter paper in the dark, and planted in 30 cm diameter pots containing sterile soil and organic fertilizer. The pots were kept in a greenhouse with a 12 h photoperiod at 25°C and with relative humidity of approximately 60–75%. The resistance was further induced by foliar spraying with 1 mM exogenous SA at 15, 30, and 45 days after sowing (DAS). At 50 DAS, six mature top leaves from two rice plants per pot were inoculated using the cutting dip method (Govindappa et al. 2011; Xu et al. 2013). After the Xoo inoculation, the rice plants were kept in a controlled temperature room with relative humidity of approximately 100% at 25°C for 24 h (Xu et al. 2013). After this period, the plants were transferred into the greenhouse. For the untreated controls, the seeds and plants were handled identically, but distilled water was used instead of SA. The BLB disease severities were recorded at 7 and 14 days after inoculation (DAI), using the scale developed by Ezuka and Horino (1974) for assessing BLB grown in a greenhouse as follows: 0 = no symptom, 1 = slight discoloration at the inoculation point, 2 = lesion is less than 15 mm long, 3 = lesion is less than ¼ of the length from the inoculated point to the leaf base, 4 = lesion is between ¼ and ½ of the length, 5 = lesion is between ½ and the whole length, 6 = lesion covers the whole length, but some green area remaining, and 7 = lesion covers the whole area. Percentage of DS was calculated using the formula reported by Li et al. (2008) as follows: DS (%) = [Sum of all numerical ratings / (Total number of leaves graded × Maximum grade)] × 100. The reduction of DS for treated rice was calculated using the formula as follows: reduction on DS = [(DS of non-treated control treatment − DS of SA-treated treatment)/DS of non-treated control treatment] × 100. The experiment was performed three times.

**Detection of superoxide anion (O₂⁻) and HR of SA-induced rice leaves**

The experiment was carried out using a CRD with five replications and five leaves per replication to detect the production of superoxide anion (O₂⁻). Leaf samples at approximately 3 cm from the leaf tip-cut were collected at 3, 6, 12, and 24 h after Xoo inoculation (HAI) (Li W et al. 2012). The O₂⁻ was detected using the Nitroblue tetrazolium staining method (Wang et al. 2009). Rice leaves from both exogenous SA-treated plants and non-treated control plants were vacuum infiltrated with 20 ml of 10 mM potassium phosphate buffer, pH 7.5, containing 10 mM NaNO₃ and 0.1% nitroblue tetrazolium (NBT, 5-bromo-4-chloro-3-indolyl phosphate, Sigma) for 24 h at room temperature. Later, the leaf samples were boiled in 95% ethanol for 60 min before visualizing the blue precipitates of O₂⁻ (Tai 2007; Lehotai et al. 2011). The leaf samples were collected at 48 HAI (Jha et al. 2007) to detect HR. The leaf samples at approximately 3 cm from the leaf tip-cut were decolorized by soaking in clear lactophenol at 65°C for 30 min and then boiled in 70% ethanol for 30 min. These samples were mounted on microscope slides with 50% glycerol in phosphate-buffered saline (PBS) and observed with a confocal microscope (Nikon, NIS Element C, Japan), using a green filter (excitation wavelength range 450 to 500 nm and detection range 515 to 565 nm) and 10× objective lens (Reimers & Leach 1991; Jha et al. 2007).

**Rapid detection of biochemical changes of SA-induced rice by Fourier transform infrared spectroscopy**

The experiment was conducted with five replications, one leaf per replication. Leaf samples of both exogenous SA-treated
and non-treated rice plants were collected at 14 DAI (Stehfest et al. 2005) and dried in a hot air oven at 60°C for 3 days, then ground by sterile pestles and mortars into fine powder. Each powdered sample was taken at an equal weight and subsequently subjected to Fourier transform infrared (FTIR) spectroscopy (Banerjee et al. 2010; Bienasantei et al. 2012) at the Synchrotron Light Research Institute, Thailand. The infrared spectra were recorded using FTIR spectroscopy (Bruker Optics Ltd., Ettlingen, Germany). The infrared spectra were collected in the 4000–900 cm\(^{-1}\) mid-infrared range at a spectral resolution of 4 cm\(^{-1}\) (Panitlertumpai et al. 2013). The individual spectrum from each group was analyzed using Principal Component Analysis (PCA) to distinguish different biochemical components of the samples by the Unscrambler \(\times\) 10.1 software (CAMO, Norway). The Savitzky-Golay method (3rd polynomial, 9 smoothing points) was employed to perform second derivative spectra, and then normalized using the Extended Multiplicative Signal Correction. Unsupervised hierarchical cluster analysis (UHCA) was performed on the second derivative spectra employing Ward’s algorithm. This method utilized a matrix to define inter-spectral distances and calculate spectra distances as D-values (Bienasantei et al. 2012).

**Assessment of Xoo growth in the rice plants**

To assess Xoo growth in plants, the experiment was carried out with five replications, one leaf per replication. The inoculated leaves of both exogenous SA-treated and non-treated rice plants were collected at 0, 1, 2, 7, and 14 DAI. Leaves were cut into 5-mm pieces and ground in sterile 10 mM MgCl\(_2\) solution, and the suspension was diluted accordingly with five replications, one leaf per replication. The inoculation was done using a water-soaked streak at a concentration of 100 CFU ml\(^{-1}\). Xoo colonies were counted after a 48 h culture at 28 ± 2°C (Hu et al. 2007; Zhao et al. 2013).

**Data analysis**

All experiments were repeated three times, with similar results in all replications. Data were analyzed and subjected to analysis of variance (ANOVA) (SPSS software, version 16). The significance of treatments was determined by the F-value using the second derivative spectra employing Ward’s algorithm. This method utilized a matrix to define inter-spectral distances and calculate spectra distances as D-values (Bienasantei et al. 2012).

**Results**

**Reduction of BLB DS by exogenous SA applications**

The inducer, 1 mM SA, was evaluated for its ability to induce resistance when used to treat seed prior to planting as well as with foliar sprays at 15, 30, and 45 DAS. The results indicate that the treatments with 1 mM SA significantly reduced the severity of BLB in the rice foliage at 7 and 14 DAI compared to the non-treated control, confirming that induction of systemic resistance had occurred. On average, the disease severities of rice plants treated with 1 mM SA were 26.19% and 38.57% at 7 and 14 DAI, respectively. These severities were significantly lower than that of the non-treated control treatment which was 34.76% and 62.38%, respectively. Disease reduction of rice plants treated with 1 mM SA was 38.17% at 14 DAI (Table 1).

### Table 1. Efficacy of exogenous SA on severity and reduction of BLB disease in rice cv. KDML 105 caused by Xanthomonas oryzae pv. oryzae under greenhouse conditions.

| Treatment            | Disease severity\(^1\) (%) | Reduction of disease severity compared with control (%) |
|----------------------|---------------------------|-------------------------------------------------------|
|                      | 7 DAI | 14 DAI | 7 DAI | 14 DAI |
| SA treated           | 26.19 ± 5.58\(^b\) | 38.57 ± 9.46\(^b\) | 24.66 | 38.17 |
| SA non-treated       | 34.76 ± 2.71\(^a\) | 62.38 ± 6.61\(^a\) |       |       |
| **Significance**     | **=** | **=** |       |       |
| **Coefficient of variation (%)** | 22.46 | 27.21 |       |       |

Rice plants were treated with seed soak and foliar spray at 15, 30, and 45 DAS, with 1 mM SA solution at pH 7 or water as a control, and inoculated with fresh X. oryzae pv. oryzae suspension at 50 DAS. The data were means ± SE with five replications, two rice plants/replication. SA treated – susceptible cultivar cv. KDML105. The reduction of BLB DS by exogenous SA applications was calculated as the percentage of disease severity in the inoculated rice plants compared to the non-treated control (SA non-treated). The results indicate that the treatments with 1 mM SA significantly reduced the severity of BLB in rice foliage at 7 and 14 DAI compared to the non-treated control, confirming that induction of systemic resistance had occurred. On average, the disease severities of rice plants treated with 1 mM SA were 26.19% and 38.57% at 7 and 14 DAI, respectively. These severities were significantly lower than that of the non-treated control treatment which was 34.76% and 62.38%, respectively. Disease reduction of rice plants treated with 1 mM SA was 38.17% at 14 DAI (Table 1).

**Expression of superoxide anion and HR of SA-treated leaves against Xoo**

The production of superoxide anion (O\(_{2}^{-}\)) was observed in rice leaves infiltrated with NBT, which specifically reacts with this reactive oxygen species (ROS) molecule. After treatment with NBT, areas with superoxide anion become dark blue in color, which can easily be visualized using light microscopy. Rice leaves treated with exogenous SA and inoculated with Xoo began to produce observable superoxide anion at 3 HAI with production gradually decreasing at 6, 12, and 24 HAI (Figure 1(a,b)). At nearly all of the time points, a statistically significant increase in the production of superoxide anion was observed for plants treated with exogenous SA. In fact, superoxide anion levels on rice leaves treated with exogenous SA and inoculated with Xoo were 0.2- to 0.6-fold higher than the non-treated rice plants (Figure 1(a,b)). In the uninoculated leaves of both SA-treated and non-treated, no blue spots were detected at any of the sampling times.

The HR was also assessed at 48 HAI by confocal microscopy which showed the changes in fluorescence of the leaf epidermis. Our results showed that the Xoo uninoculated leaves had no fluorescence; however, the inoculated leaves showed bright fluorescence. In the Xoo-inoculated group, the highest levels of fluorescence were observed on rice leaves treated with exogenous SA (Figure 2(a)). The quantification of HR showed a similar pattern as that of superoxide. Rice leaves treated with exogenous SA and challenged with Xoo showed relatively higher fluorescence than that of rice leaves treated with distilled water and inoculated with Xoo, but not statistically significant (Figure 2(b)). The HR played an important role in plant defense against bacterial infection, resulting in the appearance of necrotic lesions on leaves. BLB symptoms were pale-green to gray-green water-soaked streaks, becoming whitish or grayish from the inoculation points, and developed along the leaf margins. There was no histopathological defense reactions from the negative control, meaning the plants not inoculated with Xoo.

**Biochemical changes of exogenous SA-treated rice against Xoo**

Exogenous SA plays an important role in induction of plant defense against Xoo infection. We observed the original
average spectra and the second derivative average spectra of chemicals in the rice leaves cultivar KDML 105 in the range of 3000–2800 and 1800–900 cm\(^{-1}\) (Figure 3). The band assignments of FTIR spectra have been published in numerous publications (Table 2), but induced resistance has rarely been the focus of these studies. The FTIR peaks in our research were assigned to different functional groups in accordance with their wavenumbers (cm\(^{-1}\)) previously reported in the literature.

Second derivative spectra were applied to normalize the spectra accounting for variation in sample thickness, thus minimizing the baseline variation and providing better visual identification of bands that may overlie another in the raw spectra. Second derivative spectra in the range of 3000–2810 cm\(^{-1}\) between control and exogenous SA-treated rice leaves were compared in Figure 3(c). The broad band at the peak 2851 cm\(^{-1}\), assigned to C–H stretching vibration, was more intense in the exogenous SA treatment when compared to that of the control treatment. The comparisons between spectra of control and treated rice leaves on the range 1800–900 cm\(^{-1}\) are shown in Figure 3(d). Biochemical changes in the exogenous SA treatment showed significantly higher spectra shifts than that of the control at the vibrational peaks, 1735, 1467, and 1103 cm\(^{-1}\). These peaks can be assigned to C=O esters (peak 1735 cm\(^{-1}\)); C–H bending (peak 1467 cm\(^{-1}\)); and C–O–C glycoside (peak 1103 cm\(^{-1}\)). In addition, alpha helix structure (1647 cm\(^{-1}\)) of the amide I protein in leaves treated with 1 mM SA was changed to \(\beta\)-sheet structure (1629 cm\(^{-1}\)). In contrast, the alpha helix band remained and appeared more intense in the non-treated rice leaf (Figure 3(d)). Moreover, to further specify changes of lignin and pectin in rice leaves treated with exogenous SA, ratios of 1233/1517, 1467/1517, and 1735/1517 cm\(^{-1}\) were calculated. These biochemical component ratios of exogenous SA-treated rice demonstrated that twice as much lignin and pectin were present, and these differences were statistically significant (Figure 4).

The PCA technique was employed to analyze biochemical changes in the rice leaves treated with exogenous SA. The use of multivariate analysis, in particular the PCA, has proven useful in analysis of biospectroscopic data, providing two types of information: visualization of clustering of similar spectra of datasets in scores plots and identification of variables in loading plots. A three-dimensional PCA score plot is shown in Figure 5(a). The red points representing control treatment, although scattered widely on the right side of the graph area, can easily be distinguished from the blue points of the SA-treated one. The loading plot was used to identify variables on spectral bands that correlate with second
derivative of the average spectra. The positive loading plots at 2848, 1735, 1467, and 1107 cm$^{-1}$ are negatively correlated with the negative score plot of exogenous SA treatment. The negative loading plots at 1647, 1311, 1024, and 990 cm$^{-1}$ showed a negative correlation with the positive score plot of the non-treated treatment.

We also investigated whether a classification procedure could provide more information about the biochemical change of rice leaves treated with exogenous SA and then challenge inoculated with Xoo. To carry out such a classification, a cluster analysis was used. A dendrogram corresponding to the FTIR spectra is shown in Figure 6. The dendrogram displays two main branches: A and B. The upper main branch A is separated by 1.2 units from lower main branch B. The upper main branch A is split into two sub-groups A1 and A2, separated by approximately 0.5 units. The upper main branch A contains spectra of five replications of the treatment treated with exogenous SA. The spectra within the lower main branch B are five replications of the SA-non-treated control treatment. Within this lower main branch B, the heterogeneity between spectra is approximately 0.2 units.

Table 2. Band assignments of FTIR vibration peaks (cm$^{-1}$) of plant rice leaf tissue based on literature references.

| Peak name                      | Spectral ranges | Vibration peak assignments                                                                 | References                                                                 |
|--------------------------------|-----------------|------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------|
| C–H asymmetric and symmetric stretching vibration | 3000–2800       | C–H asymmetric and symmetric stretching vibration of mainly lipid groups with the little contribution from protein | Jouraiphy et al. (2008), Iqbal et al. (2009), Mularczyk-Oliwa et al. (2012), Sivakumar et al. (2014), Lahlali et al. (2015) |
| C=O esters                     | 1740–1700       | Stretching vibration of C=O ester of bond lipid, lignin, pectin, or their esters               | Sene et al. (1994), Kenneth and Lawrence (2005), Dokken and Davis (2007), Iqbal et al. (2009), Lahlali et al. (2015) |
| Amide I                        | 1700–1600       | Amide I due to C=O stretching of α-helix protein, contribution from C–N stretching (C=O stretch (80%), C–N stretch (10%), N–H bending (10%)) | Sene et al. (1994), Yu et al. (2003), Ellepola et al. (2005), Jouraiphy et al. (2008), Sivakumar et al. (2014), Lahlali et al. (2015) |
| Amide II                       | 1600–1500       | Amide II due to N–H bending and C–N stretching of protein (N–H bend (60%), C–N stretch (40%)) | Sene et al. (1994), Wetzel and LeVine (2000), Jouraiphy et al. (2008), Sivakumar et al. (2014) |
| C=O aromatic ring               | 1517            | C=O aromatic ring from lignin, C–H bend                                                      | Sene et al. (1994), Wetzel and LeVine (2000), Mularczyk-Oliwa et al. (2012), Wang et al. (2012), Lahlali et al. (2015) |
| C–H bending                    | 1470–1350       | C–H bending from CH2 and CH3 from mainly lipids and lignin                                    | Sene et al. (1994), Kenneth and Lawrence (2005), Kumar and Min (2011) |
| C-O Stretching hemicellulose and lignin | 1300–1200     | C–C, C–O skeletal                                                                            | Alonso-Simon et al. (2004), Mularczyk-Oliwa et al. (2012) |
| C–C ring cellulose             | 1155            | C–C ring from cellulose                                                                       | Sene et al. (1994), Kacurakova et al. (2000), Alonso-Simon et al. (2004) |
| C=O–C glycoside                | 1103            | C=O–C glycoside ether mainly hemicellulose                                                    | Dokken and Davis (2007)                                                   |
| C, C–C stretching              | 1022, 1047, 1080 | Stretching vibration of C–OH of alcoholic groups and carboxylic acid, C–C bond of the cellulose sugar rings. Mainly C–O–C of polysaccharides | Sene et al. (1994), Kacurakova et al. (2000), Alonso-Simon et al. (2004), Iqbal et al. (2009), Suehara et al. (2012) |
The reduction in BLB severity in rice treated with exogenous SA could result from the increased production of $\text{O}_2^-$. The nitroblue tetrazolium (NBT) staining compound reacted with $\text{O}_2^-$ to form a dark insoluble formazan compound and precipitated in the reactive rice leaf area, forming dark blue spots. In the non-inoculated leaves of all treatments, no blue spots were detected in the leaf tissues at any sampling time, indicating that $\text{O}_2^-$ was maintained at the cellular redox balance in the plant cell. In our study, the defence mechanisms of rice cells treated with 1 mM SA were systemically activated and enhanced, prior to challenge inoculation with Xoo. Our results showed that, after inoculation with Xoo, rice leaves treated with exogenous SA produced 28% more $\text{O}_2^-$ than that of the control treatment. The pattern of $\text{O}_2^-$ production in response to temperature stress and pathogen infection is species specific (Dring 2006), but generally the production of $\text{O}_2^-$ is rapid, intense, and short-lived. It is known that, after induction, $\text{O}_2^-$ is immediately released at the external surface of the membrane (Auh & Murphy 1995). The $\text{O}_2^-$ not only has direct toxicity to pathogens, but it is also the central component of the plant defence signal transduction pathways leading to HR, cell wall reinforcement, SA synthesis, and defence gene expression (Lamb & Dixon 1997; Lehotai et al. 2011; Graves 2012; Sharma et al. 2012; Pastor et al. 2013). $\text{O}_2^-$ is converted into $\text{H}_2\text{O}_2$, a substrate for peroxidase that mediates production of plant cell wall components such as cellulose, lignin, pectin, and callose (Carpin et al. 2001). The rapid rise of $\text{O}_2^-$ leads to HR (Lamb & Dixon 1997), another marker of resistance characterized in our study. HR can be evaluated by tracking the excitation of phenolic compounds at 488 nm using confocal microscopy where increase in fluorescence indicates accumulation of these defence compounds (Czymmek et al. 2002). In this study, the HR was observed on rice leaves at 36–48 HAI with Xoo (Jha et al. 2007). Our results showed that rice leaves treated with exogenous SA had 110% more HR than the control at 48 HAI. As such, application of SA improved the HR, leading to increased formation of zones of rice dead cells around infestation zones to stop Xoo spread and BLB disease development.

The expression of the defence biomarkers, $\text{O}_2^-$ and HR, after Xoo challenge inoculation was similar to their induction in soybean, sweet orange, and cassava by inducers including SA, chitosan, Bacillus amyloliquefaciens KPS46, and B. subtilis CaSUT007 (Shirasu et al. 1997; Buensanteai et al. 2009; Coqueiro et al. 2015; Thumanu et al. 2015). Since plants rely on reinforcement of the cell wall to resist infection, this study focused on characterizing the changes in the composition of cell wall constituents in exogenous SA-treated rice using FTIR spectroscopy. Most of peaks of FTIR spectra in our study were similar to those of the report of Sene et al. (1994) in non-treated rice plants. Priming and inducing of plant defences using these inducers results in increased ability of the plant to reinforce the plant cell wall by altering its composition, primarily with lignins, pectins, amide I structure, and lipids. (Banerjee et al. 2010).

The composition of polysaccharides in the cell wall is an important factor in host–pathogen interactions, resulting in differential plant disease responses (Vorwerk et al. 2004; Miedes et al. 2014). Polysaccharides from plant cells, when degraded into oligogalacturonides, could elicit defence responses in plants (Shibuya & Minami 2001). Our results showed that rice leaves treated with 1 mM SA and challenge growth in the rice plants

A distinct difference in population sizes of Xoo began to be observable at 1 DAI between SA-treated leaves and control leaves. The population size of Xoo in rice leaves treated with exogenous SA reached maximal growth approximately $4.4 \times 10^5$ cfu per leaf at 7 DAI, while the Xoo population in rice leaves treated with distilled water continued to multiply and reached a maximal population approximately $5.6 \times 10^5$ cfu per leaf at 14 DAI. The Xoo population size on rice leaves treated with SA at 14 DAI was half the size of those treated with distilled water (Figures 7 and 8), suggesting that growth of Xoo was inhibited by application of exogenous SA. A proposed schematic model of systemic resistance in rice plants against Xoo after being treated with exogenous SA is shown in Figure 8.

**Assessment of Xoo growth in the rice plants**

**Discussion**

The objective of this study was to characterize rice responses to SA and Xoo inoculation in association with induced resistance using analysis of $\text{O}_2^-$, HR, and biochemical composition including lignin, pectin, amide I structure, and lipids (Lamattina & Polacco 2007; Buensanteai et al. 2009; Pastor et al. 2013). Treatment of rice seeds and leaves with exogenous SA resulted in changes in these resistance markers. Treating rice plants with exogenous SA reduced the BLB severity significantly compared with that of the non-treated control. The reduction was 24.66% when assessed at 7 DAI, but it was up to 38.17% at 14 DAI. This finding was similar to that of Nagendran et al. (2013) who found that endophytic bacteria could reduce the BLB severity approximately 3.42–39.82%. In addition to controlling BLB severity, exogenous SA has also been shown to control rice blast. At 8 and 10 mM concentrations, a foliar spray could reduce blast severities by 73.9% and 61.5%, respectively (Daw et al. 2008). In addition, Al-Hakimi (2006) reported that application of 0.6 mM SA enhanced pectin and lignin in soybean plant against drought stress.

**Figure 4.** Relative absorbance ratio of several spectral peaks to the intensive at 1517 cm$^{-1}$ in KDML 105 rice leaves treated or non-treated with 1 mM SA and challenge inoculated with Xoo, at 14 DAI, under greenhouse conditions. Error bars represent standard deviation of six replications. Values followed by the same letter are not significantly different according to Duncan’s multiple range test at $P=0.05$. Ratio of 1233/1517 cm$^{-1}$ represents methoxyphenolic substitution in aromatic units of lignin. Ratio of 1467/1517 cm$^{-1}$ is the ratio of syringyl to guaiacyl (S/G) of lignin. Ratio of 1735/1517 cm$^{-1}$ is representative of an alteration in pectin synthesis.
inoculated with Xoo had a higher peak C–O–C glycoside at 1103 cm\(^{-1}\), when compared to rice leaves treated with distilled water and challenge inoculated with Xoo. For polysaccharides detected within the range of 1200–900 cm\(^{-1}\), our results showed that only one peak at 1103 cm\(^{-1}\) was more intense in the SA treatment while other peaks were intense in the control treatment. These results are similar to the FTIR analysis of Thumanu et al. (2015) where epidermal cells of cassava leaves were treated with the biotic inducer \textit{B. substilis} CaSUT007. The strain, \textit{B. substilis} CaSUT007, induced resistance in cassava against casava anthracnose disease. Some questions remain as to why most peaks of treated plants in the range of polysaccharides from 1200 to 900 cm\(^{-1}\) were less intense than the non-treated control, indicating relatively decreased polysaccharide production. Rice plants treated with exogenous SA may rearrange their polysaccharides to synthesize some specific products for disease resistance such as defence-related enzymes, PR-proteins, lignin, and pectin. Alteration of lignin monomer composition during disease resistance has been reported (Faix 1991; Barber et al. 2000; Martin et al. 2005; Menden et al. 2007). The higher ratios of 1233/1517, 1467/1517, and 1735/1517 cm\(^{-1}\) wavelengths in our results are in line with the research of Martin et al. (2005). In their study, changes in these ratios corresponded with resistance of Dutch elm plants; specifically, the ratios were approximately 20–33% higher than those of susceptible ones.

Lignin is characterized by the ratios of 1233/1517 and 1467/1517 cm\(^{-1}\). The ratio of 1233/1517 cm\(^{-1}\) represents methoxyphenolic substitution in aromatic units of lignin.

Figure 5. PCA analysis in KDML 105 rice leaves treated or non-treated with 1 mM SA and challenge inoculated with Xoo at 14 DAI under greenhouse conditions. (a) 3D scatter plot of score from PCA analysis. (b) PC1 loading plot from PCA analysis in the range of 3000–2810 and 1800–900 cm\(^{-1}\). (c) PC3 loading plot from PCA analysis in the range of 3000–2810 and 1800–900 cm\(^{-1}\).
increased lignification is reflected in cucumber and pepper by enhanced APX (Wu et al. 1997; Ray et al. 1998; Zheng et al. 2005), and in potato by PAL (Henderson & Friend 1979). In tomato, exogenous application of 0.01% SA enhanced resistance to *Ralstonia solanacearum*, in part, through a greater accumulation of lignin in roots upon infection (Mandal et al. 2013).

In addition to lignification, alteration in pectins also contributes to plant defence against BLB disease. The ratio of 1735/1517 cm$^{-1}$ is representative of an alteration in pectin synthesis (Chatigjakis et al. 1998). Enrichment of pectins strengthens the plant structural barrier properties of cell walls during the colonization stage by the pathogen (Cherif et al. 1991; Eckardt 2002; Raiola et al. 2011; Volpi et al. 2011; Bethke et al. 2014). We suggest that the exogenous SA reduces the *Xoo* bacterial colonization in the rice leaf tissues initially by inducing reinforcement of structural barriers such as thicker cell walls as occurs in chitosan-treated tomato plants (Benhamou, 1996).

In Gramineae species, the cell is poor in protein (Sene et al. 1994; Wang et al. 2012). Our results showed that the amide I band underwent a structural change which was detected in the range of 1700–1600 cm$^{-1}$. The alpha helix structure of proteins was converted to a $\beta$-sheet structure (1629 cm$^{-1}$) in leaves treated with 1 mM SA. Amide I $\beta$-sheet structure has been shown to be involved in the resistance of plants to pathogens (Thumanu et al. 2015). The Amide I $\beta$-sheet secondary structure of the small peptide molecules had a major role in plant against stress responses (Dubovskii et al. 2011; Tavormina et al. 2015). These authors suggested that the amide I $\beta$-sheets of small peptides cause cysteines of different domains to form disulfide bonds, leading to a new stable fold structure. The altered structure provided a high affinity binding site for plant receptors to transduce defence signals produced by pathogen and other stress responses (Dubovskii et al. 2011; Tavormina et al. 2015).

Lipids can be identified using the peaks at 2851.3 cm$^{-1}$ (CH$_2$ stretching vibration) and 1735 cm$^{-1}$ (C=O esters). We observed more intense peaks at these wavelengths in leaves treated with 1 mM SA and inoculated with *Xoo* relative to the control. In 2002, Feussner and Wasternack reported that formation of oxygenated fatty acids, abbreviated as oxylipins, was the response of plant cells against abiotic and biotic stress. Oxylipins directly act as antimicrobial compounds and indirectly stimulate defence gene expression (Farmer et al. 2003). Additionally, two groups of galactolipids have roles during the induction of systemic resistance. Plant digalactosyldiacylglycerol (DGDG) contributes to the production of nitrite oxides as well as SA biosynthesis, and it is required for the induction of SAR. In contrast, plant monogalactosyldiacylglycerol (MGDG) controls the biosynthesis of the SAR signals azelaic acid (AzA) and glycerol-3-phosphate (G3P) (Gao et al. 2014).

One of the most important findings of this study is the suppressive effect of exogenous SA applications to rice on *Xoo* population growth. The results of Zhang et al. (2013) on putative virulence-relevant genes of *Xoo* in rice showed that *Xoo* population was approximately $10^3$ to $10^4$ cfu per leaf at 1 and 2 DAI. Beside, transgenic expression of the gene *Xa21* in rice resulted in a decrease in the *Xoo* population by approximately $10^3$ to $10^4$ cfu per leaf at 1 and 2 DAI relative to the control (Peng et al. 2015). Maximum *Xoo* population in rice leaves is usually recorded at 12–15 DAI (Hu et al. 2001). Additionally, 1467/1517 cm$^{-1}$ is the ratio of syringyl to guaiacyl (S/G) monomers of lignin (Faix 1991). In our research, the higher S/G ratio occurred in leaves treated with 1 mM SA relative to the control treatment, indicating that higher synthesis of syringyl monomers occurred in treated leaves. The S unit of lignin has been shown to accumulate in resistant wheat leaves during the HR response (Bishop et al. 2002; Menden et al. 2007). Changes in the S/G ratio can be explained by changes in the activity of defencerelated enzymes such as phenylalanine ammonia-lyase (PAL) (Chen & McClure 2000; Gayoso et al. 2010). PAL catalyses and regulates the production of precursors for lignin biosynthesis in plant cells (Nicholson & Hammerschmidt 1992). When the *Xoo* tries to penetrate the plant cell wall, the rice cell wall initiates dynamic structural and compositional changes (Eggert et al. 2014), including lignification (Nicholson & Hammerschmidt 1992; Carver et al. 1998; Zhao & Dixon 2014). Lignin is a difficult polymer for *Xoo* to degrade (Nicholson & Hammerschmidt 1992). The
et al. 2007; Zhao et al. 2013; Chen et al. 2014). Apoplastic water potential decreases during the process of HR inhibit growth of bacterial pathogens (Wright & Beattie 2004).

From this study and literature data, we propose a simple model of systemic resistance (Figure 8). In this model, SA is bound by plant receptors on the plasma membrane such as GTP-binding proteins or SA-binding proteins, leading to changes in receptor conformation that result in activation of kinases and ion channels (Klessig et al. 2000; Kumar & Klessig 2003; Chen & Ronald 2011). Rice contains seven genes encoding Rac GTPases which activate protein kinases (MAPK cascades). MAPK cascades could transmit extracellular signals to downstream components through protein phosphorylation. Seventeen MAPKs have been identified in rice (Ichimura et al. 2002; Chen & Ronald 2011). A MAPKK phosphorylates a serine or threonine residue on a MAPKK, which in turn, activates a MAPK by the dual phosphorylation of a threonine and tyrosine residue (Cakir & Kilickaya 2015). Activated effectors continue to transfer the signals of molecule SA to secondary messengers which could amplify the signals and send them to other reactions (Rivas & Thomas 2005). As a result of signal transduction, a series of defence responses are induced, including increased ion fluxes, extracellular alkalization, cytoplasmic acidification, nicotinamide adenine dinucleotide phosphate oxidase activation, oxidative burst, ROS production, HR, phosphorylation, and early defence reactions (Koornneef et al. 2008; Kepczynska & Kro 2012; Mishra et al. 2012; Chinta et al. 2015; Al-Issawi et al. 2016).

A long distance SA signal stimulates late defence genes and secondary metabolite accumulation (Koornneef et al. 2008; Mishra et al. 2012). Additionally, ion fluxes conclude Ca$^{2+}$ and H$^+$ influx, K$^+$ and Cl$^-$ efflux, which are immediate responses of plant cells leading to extracellular alkalization and cytoplasmic acidification (Boller 1995; Mishra et al. 2012; Pastor et al. 2013). Another common early defence event in plant cells is the oxidative burst and production of ROS (Albert et al. 2006). The presence of ROS may result from the transfer of one, two, and three electrons, respectively, in the reduction of molecular O$_2$ from predominantly superoxide anion (O$^-$), peroxyhydrogen (H$_2$O$_2$), and hydroxyl (HO$^-$) (Mittler 2002; Mishra et al. 2012). ROS does not only have direct toxicity to pathogens, but it also the central component of plant defence signal transduction pathway leading to the HR, cell wall reinforcement, and defence gene expression (Pastor et al. 2013). The cell death both inhibits the invasion of bacterial pathogens and stimulates production of SA. SA can directly affect the pathogens and act as a signal of SAR (Prakongkha et al. 2013; Buensanteai et al. 2014). The SA signal stimulates defence genes involved in cell wall modification, PR proteins, and production of secondary metabolites in plants (Stintzi et al. 1993; Taguchi et al. 2001; Mishra et al. 2012).

Plant cell walls undergoing pathogen attack typically initiate the formation of structural barriers utilizing lignin and pectin (Sherif et al. 1991; Nicholson & Hammerschmidt 1992; Carver et al. 1998; Quiroga et al. 2000; Eckardt 2002; Al-Hakimi 2006; Raiola et al. 2011; Volpi et al. 2011; Mandal et al. 2013; Bethke et al. 2014; Zhao & Dixon 2014). Secondary metabolites including phenolic compounds, phytoalexins, and compounds involved in HR are important to plant defence (Mishra et al. 2012). Inducers of SAR help treated plants to be resistant to a broad spectrum of pathogens. When rice plants are inoculated with Xoo after SA treatment, the host more quickly recognizes the infection, creating a stronger signal transduction pathway with resulting ion fluxes, ROS, cell wall fortification, phytoalexins, and long-distance signal production. This explains our observation that rice plants treated with SA have decreased DS relative to controls.

**Conclusion**

Induced resistance in the susceptible rice variety KDML 105 to Xoo by exogenous SA treatment was characterized by various known defence responses including increased O$^-$

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**Figure 8.** A proposed schematic model of systemic resistance in rice plant against Xoo after being treated with exogenous SA. Briefly, the molecule of exogenous SA would be attached by plant receptors such as GTP-binding proteins or SA-binding protein, leading to change in receptor conformation and resulting in activation of kinases and ion channels. Activated effectors continue to transfer the signals of molecule SA to secondary messengers which could amplify the signals and send them to other reactions. Following pathogen recognition and signal transduction, a series of defence reactions occur including an increase of ion fluxes, ROS production, phosphorylation of proteins, phytoalexin synthesis, and inductions of secondary metabolite pathways. SA behaves as a long-distance signal and stimulates defence genes. Upon inoculation, SA-treated rice plants could quickly recognize the Xoo, leading to stimulation of HR and cell death, which isolate the pathogen infection. Rice plants could fortify cell walls, producing more phytoalexins and systemic signals.
production, enhanced HR, lipid production, cell wall modifications, and protein structural changes. These defence responses together provided effective suppression of the Xoo population on leaves. In order to further characterize pathways involved in the induced defence response and to compare it to the response of other plant species, we plan to study the transcriptome and proteome of rice plants treated with exogenous SA and inoculated with Xoo. Moreover, an appraisal of effectiveness under the field conditions should be performed in order to evaluate exogenous SA application as a practical management strategy of the BLB disease. These findings will help researchers to better understand induced resistance in rice and will help farmers to protect rice yields from losses related to disease infection.

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Disclosure statement
No potential conflict of interest was reported by the authors.

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