Hue University Journal of Science: Natural Science; ISSN 1859-1388

Vol. 127, No. 1D, 2018, P. 63-72; DOI: 10.26459/hueuni-jns.v127i1D.5063

FOURIER TRANSFORM INFRARED CHARACTERIZATION OF SALICYLIC ACID TREATED RICE PLANTS AGAINST *Xanthomonas oryzae* pv. *oryzae*

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Abstract. Salicylic acid (SA) plays an important role in induction of plant defence against a wide range of biotic and abiotic stresses through physiological, biochemical and molecular responses. The study was carried out to evaluate defence reponses of SA- induced rice plants against leaf blight (LB) disease, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). In this study, a series of SA solutions at concentrations of 0.25, 0.50, 0.75, 1.0, 2.0 and 4.0 mM were evaluated for their potential to protect rice LB in the net house conditions. Biochemical analyses were characterized by means of a FTIR spectroscope. Our results showed that at inoculated leaves, the elicitor of SA at a concentration of 1.0 mM had highest reduction of disease severity approximately 34 % at 21 days after inoculation, compared to the control. Moreover, SA application and *Xoo* inoculation were efficient for enhancing of carbohydrates, lipids and proteins modifications at both leaves above and below the *Xoo*-inoculated leaf. These results collectively suggest that the elicitor of SA strongly induced the systemic resistance in rice plants.

Keywords: biochemical modification, induced resistance, rice, salicylic acid

1 Introduction

Rice plants face a range of enviromental challenges including temperature, drought, pathogen and insect attacks. Rice leaf blight (LB) caused by *Xanthomonas oryzae* pv. *oryzae* Ishiyama (*Xoo*) widely occurs in Thailand and Viet Nam due to favorable warm and wet climate conditions [1, 2]. The pathogenic *Xoo* causes yellowish-white lesions with wavy edges on leaf tips and margins [3]. A severe infection could cause whitish or greyish blight in all infected leaves, resulted in death of leaves and tillers, affecting yield loss in rice of approximately 20-75% [1, 3]. Chemical control using bactericides such as copper oxychloride, streptomycin, tetracylin, copper hydroxide, copper sulfate, quarternary ammonium compound, hydroxyquinoline sulfate, has been mainly recommended to LB control but the application is often ineffective due to continuous rainfall during the rainy season [4]. Apart from such limitations, the target *Xoo* bacterium

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Submitted: 29-11-2018; Revised: 5-12-2018; Accepted: 29-12-2018

could build up its resistance ability to bactericides regularly used, making the chemical application less desirable. Moreover, the bacterides always leave their residues in rice plants and environment.

In recent years, resistance elicitors or inducers have been evaluated to control plant diseases based on the induced resistance concept. The induced resistance is a part of the plant innate defense system that could confer a long-lasting protection against a broad range of plant pathogens [5]. Once the disease resistance inside plants is induced, the plants acquire an enhanced defensive capacity against further infections by plant pathogens. The defense system is multi-layers and comprises constitutively inducible defense means including biochemical modifications. The resistance elicitors could be both synthetic and natural compounds. The elicitors such as salicylic acid (SA), sodium saccharin dihydrate, 2,6-dichloroisonicotinic acid, antimycin A, riboflavin, chitosan, acibenzolar-S- methyl, have been assessed on their efficacy against several plant diseases, mostly in fungal pathogens [6, 7, 8]. Mechanisms of these elicitors on induced resistance have been recently revised and include a rising of cytosolic H^+ and Ca^{2+}, an activation of MAP-kinases, a callose deposition, an oxidative burst, a hypersensitive response, a synthesis of defense enzymes, an accumulation of jasmonate, salicylic acid, phytoalexins and pathogenesis-related proteins [9, 10]. Even though there have been proves of effectiveness and mechanism on using SA for controlling crop diseases, none of research has been characterize differences of defensive carbohydrates, proteins and lipids at various plant leaf positions. Recently, there is a novel approach using Fourier transform infrared (FTIR) spectroscopy to analyze biochemical traits of induced plants. The FTIR technique allows for the analysis of chemical bonds of carbohydrates, proteins and lipids. Applications include the tracing of biochemical alterations that may originate from interactions of high relevance in various pathological processes between pathogenic *Xoo* and induced rice plants.

The present study was aimed to further explore biochemical modifications of different SA-induced rice leaves with respect to an infection of pathogenic *Xoo*. The effects were analyzed using the FTIR technique.

2 Materials and methods

2.1 Salicylic acid preparation

A solution series of SA (Acros Organics, ThermoFisher Scientific, USA) was prepared according to treatment descriptions. To assure complete solubility, each solution was stirred with a magnet bar for at least 1 h and also vigorously shaken before its application. Sterile distilled water was used as the non-treated control.

2.2 Culture of pathogenic bacteria

The virulent strain SUT1-121 of Xoo was obtained from the Plant Pathology and Biopesticide Laboratory, Suranaree University of Technology, Thailand. The Xoo was retrieved by streaking it onto nutrient glucose agar (NGA) at 28 ± 2°C for 48 h. Then, the bacterial cultures were propagated in 300 ml of nutrient broth containing 2% glucose (NGB) culture medium at 28 ± 2°C with a constant shaking at 180 rpm, for 48 h. Finally, the bacterial cultures were re-suspended in sterile distilled water and a density of the suspension was adjusted to 1x108 cfu ml-1 based on an optical density of 0.2 at 600 nm.

2.3 Preparation of experimental rice plants and application of SA

Seeds of the rice cultivar KDML 105, a susceptible variety, were obtained from Organic Farm, Suranaree University of Technology, Thailand. To determine whether treatment of SA had efficacy on induced resistance, seeds of KDML 105 were treated with a solution series of SA including 0.25, 0.50, 0.75, 1.0, 2.0 and 4.0 mM. The susceptible rice seeds were surface-disinfected by a treatment with 95% ethanol (v/v) for 2 min, followed by five washes with sterile distilled water to remove the alcohol residue, then soaked in sterile distilled water overnight. Subsequently, 30 g of the rice seeds were soaked in 50 ml of a solution of SA, germinated on wet filter papers in the dark conditions for 12 h [11], and planted in 40-cm-plastic pots containing sterile soil and organic fertilizers. Pots were kept in a net house with 12 h photoperiod at 25 °C and relative humidity at approximately 60-75 %. Rice plants were watered every two days, supplemented with mineral fertilizers as needed in net house conditions to avoid any stress due to insects or pathogenic agents. Rice plants were further induced by foliar sprays of each solution of SA at 15, 30 and 45 days after planting (DAP). Fifty-day-old rice plants were used for the study [12].

2.4 Plant inoculation and LB disease assessment

At 50 DAP, six matured top-leaves from each pot were randomly chosen and inoculated by cuting leaf at 3 cm from the leaf tip, then inoculating with Xoo suspension at a density of 1x108 cfu mL−1 [12, 14] (Figure 1). After the Xoo-inoculation, the rice plants were covered by black plastic bags, and incubated in an inoculation room for 24 h [13, 14]. Then, rice plants were transferred into a net house.

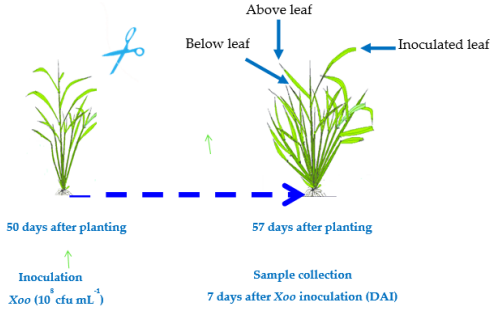

Fig. 1. The experimental procedure. *Seed soak and foliar sprays with SA in rice cv. KDML105 grown under a nethouse conditions at 0, 15, 30 and 45 days after planting.*

LB disease severity scale was recorded at 14 and 21 days after Xoo inoculation (DAI) [13], using a modified disease scale by IRRI [15] for assessing LB lesions under net house conditions as follows 0 = no symptom, 1 = slight discoloration at the inoculation point, 2 = blight lesion is less than 15 mm long, 3 = blight lesion is less than ¼ of the length from the inoculation point to the leaf base, 4 = blight lesion is between ¼ and ½ of the leaf length, 5 = blight lesion is between ½ and the whole leaf length, 6 = blight lesion covers the whole leaf length, but some green area remaining and 7 = blight lesion covers the whole leaf. Then, a percentage of disease severity was calculated as follows Disease severity (DS, %) = [Sum of all numerical ratings / (Total number of leaves graded * Maximum grade)] * 100%. The experiment was carried out with five replications, two rice plants per one replication.

2.5 Biochemical imaging and FTIR data analysis

The samples of above and below rice leaves (Figure 1) were collected at 7 DAI and dried in a hot air oven at 60 oC for 3 days. Next, leaf samples were ground by sterile mortars and pestles into fine powder, then immediately subjected to the infrared measurements. This experiment was carried out at Plant Pathology and Biopesticides Laboratory, Suranaree University of Technology and Synchrotron Light Research Institute, Thailand. Infrared absorption spectra were recorded by means of a FTIR spectroscope (Bruker Optics Ltd., Ettlingen, Germany). The spectra were collected in the mid-IR range of 4000-900 cm-1 at a resolution of 4 cm-1. Twelve spectra, each averaged over ten scan, from each sample was corrected for background spectrum, displayed in the absorbance mode and analyzed using OPUS software (Cooperative Library Network Berlin-Brandeburg) and Unscrambler x10.1 software (CAMO, Norway). Preliminary spectral analyses were conducted with the OPUS software from the manufacturer instruction. Second derivative spectra were calculated in 9 smoothing points of 3rd polynomial mode by the Savitzky-Golay algorithm [14]. Chemical functional groups of carbohydrates, proteins and lipids were identified according to previous published reports.

2.6 Reproducibility of experiments and statistical analyses

The presented data were resulted from single biological experiment. Results were repeated 3 times in biologically independent experiments with similar outcomes. The experiments were carried out with completely randomized design. Data were analyzed and subjected to Analysis of Variance (ANOVA) using SPSS software, version 16. The significance of treatments was declared by the magnitude of F value at P = 0.05. Treatment means were separated by Duncan's Multiple Range Test (DMRT).

3 Results

3.1 Efficacy of salicylic acid on induced resistance against rice LB disease under net house conditions

In this experiment using rice cultivar cv. KDML105, seed and foliar treatments with SA reduced the severity of LB in the rice foliage, confirming that an induction of systemic resistance had

occurred. The results indicated that high concentrations of SA inhibited disease severity better than the low concentrations, including 0.25 and 0.50 mM. The results also showed that at 14 and 21 DAI, disease severities of rice plants treated with 1.0 mM SA using a seed soak and foliar sprays were significantly lower than those of other SA-treatments and the non-treated control. Moreover, our results revealed that on the observing time point at 21 DAI, treatment of SA at a concentration of 1.0 mM had low disease severity at 25.71 %, and significant difference to the control treatment at approximately 39.05 % (Table 1).

The elicitor of SA at a concentration of 1 mM was used to further characterize its ability on induced resistance at two rice leaf positions, including leaves above and below the Xoo-inoculated leaf.

Table 1. The efficacy on induced resistance of salicylic acid at different concentrations to leaf blight disease in rice under net house conditions

Treatment of SA (mM)	Disease severity [1/] (%)		Reduction of disease severity (%)	
	14 DAI [2/]	21 DAI [2/]	14 DAI [2/]	21 DAI [2/]
0.25	30.48±3.10^{ab}	34.76±3.19^{ab}	8.55	10.99
0.50	29.52±2.92^{abc}	33.33±3.91^{b}	11.43	14.65
0.75	28.57±4.33^{bc}	32.38±3.61^{b}	14,28	17.08
1.0	22.86±3.61^{d}	25.71±3.53^{c}	41.41	34.16
2.0	25.24±.505^{cd}	26.19±3.10^{c}	24.27	32.56
4.0	26.19±3.10^{bcd}	26.67±1.30^{c}	12.42	31.34
0.0 (Control)	33.33±4.12^{a}	39.05±8.00^{a}		
F-test	*	*		
CV (%)	5.68	7.57		

[1/] Mean ± SE (standard error) followed by the same letter do not differ significantly according to Duncan's multiple range test at P = 0.05; [2/] DAI: Days after inoculation

3.2 Biochemical characteristics between two rice leaf positions

Second derivative average spectra of rice leaves of the SA-induced treatment and diseased control in the range of both 3000-2800 cm^{-1} and 1800-900 cm^{-1}, were shown in Figure 2. The assignment of specific FTIR general peaks have been published in numerous publications, but the study on induce resistance is not much (Table 2). Second derivative spectra in the range of 3000-2800 cm^{-1} between the control and exogenous SA-treated rice leaves at two leaf positions were similar, shown in Figure 2A and 2C. In the wavelength range of 1800-900 cm^{-1}, at above rice leaves, its FTIR peaks of the SA-induced treatment had significantly higher spectra shift than that of the control at only one vibrational peak at 1734 cm^{-1} (as assigned to C=O esters) (Figure 2B). At below rice leaves, biochemical changes in the exogenous SA treatment showed significantly higher spectra shifts than those of the control at some vibrational peaks, including 1736 cm^{-1} (C=O esters), 1103 cm^{-1} (C-O-C glycoside) and 1040 cm^{-1} (C-C, C-O stretching). Moreover, alpha helix structure at 1655 cm^{-1} of the amide I protein in leaves treated with 1 mM SA was changed to β-sheet structure at 1636 cm^{-1}. In contrast, the alpha helix band remained and appeared more intense in the non-treated rice leaves (Figure 2D). The results showed that above and below rice leaves have various defense responses on carbohydrates, lipids and proteins. The defensive activity at the below leaves is more abundant than that of the above leaves.

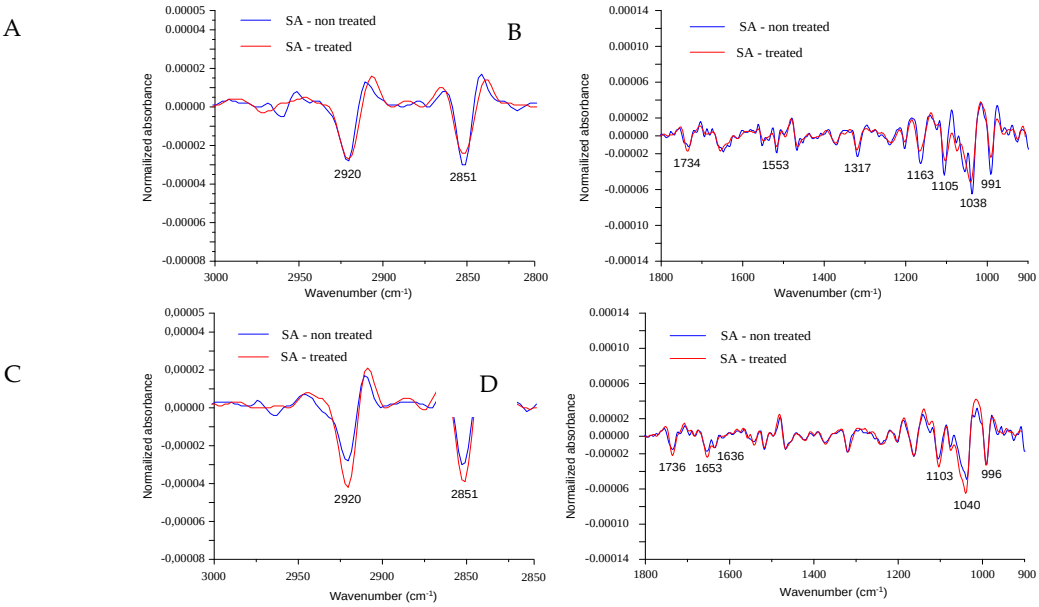

Fig. 2. Second derivative average spectra in KDML 105 rice leaves treated with 1 mM SA and inoculated with *Xoo*, at 7 DAI, under net house conditions. A and B: *Second derivative average spectra in the range of 3000-2800 and 1800-900 cm^{-1} in leaves above the Xoo-inoculated leaf; C and D: Second derivative average spectra in the range of 3000-2800 and 1800-900 cm^{-1} in leaves below the Xoo-inoculated leaf.*

Table 2. Band assignments of FTIR vibrational peaks (cm^{-1}) of rice leaf tissues

Peak name	Spectral ranges	Vibrational peak assignments	References
C-H stretching vibration	3000-2800	C-H Asymmetric and Symmetric stretching vibration of mainly lipid groups	[16, 17, 18]
C=O esters	1740-1700	Stretching vibration of C=O ester of bond lipid, lignin, pectin or their esters	[16, 19]
Amide I	1700-1600	Amide I due to C=O stretching of α-helix protein	[16, 18]
C-O Stretching lignin and hemicellulose	1300-1200	C-C, C-O skeletal	[17, 20]
C-O-C glycoside	1103	C-O-C glycoside ether mainly hemicellulose	[19]
C-C , C-O Stretching	1022, 1047, 1080	Mainly C-O-C of polysaccharides	[20, 21]

Two-dimensional PCA analyses in above and below rice leaves were presented on Figure 3. The blue points representing control treatment, scattered widely on the right side of the graph area, could easily be discriminated from the red points of the SA-treated one, with both above and below rice leaves (Figure 3A and 3B).

Moreover, to further characterize changes of lignin and pectin in rice leaves treated with exogenous SA, three ratios of 1233/1517 cm^{-1}, 1467/1517 cm^{-1}, and 1735/1517 cm^{-1} were calculated. These biochemical component ratios of exogenous SA-treated rice at above leaves were significantly higher than those of the control one. Meanwhile, these biochemical component ratios of below leaves were on the contrary to results of the above leaves (Figure 4A and 4B).

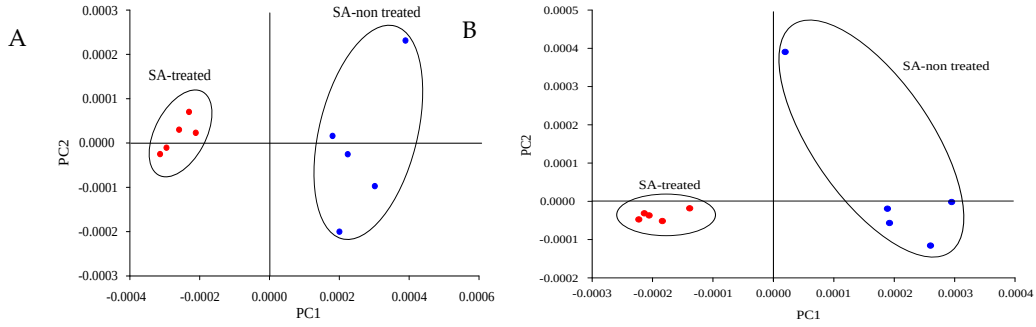

Fig. 3. 2D scatter plot of score from PCA analysis of rice leaves, at 7 DAI, under net house conditions. *A: above rice leaves; B: below rice leaves*

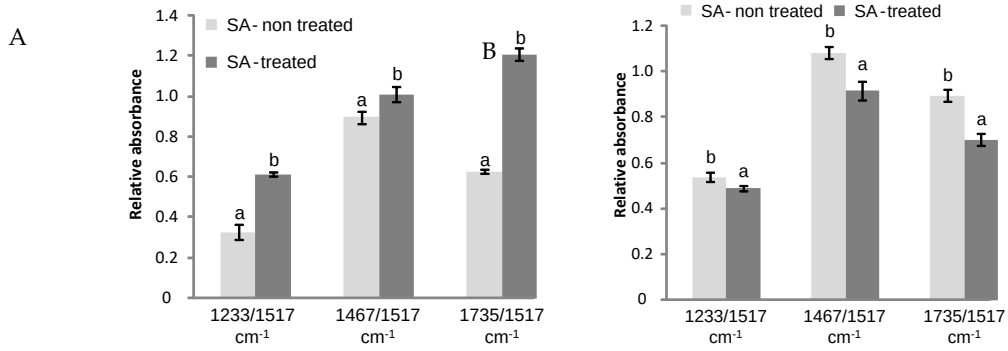

Fig. 4. Relative absorbance ratios of some spectral peaks to the intensitive at 1517 cm^{-1} in KDML 105 rice leaves treated or non treated with SA 1mM and inoculated with *Xoo*, at 7 DAI, under the net house conditions. *Error bars represent standard deviation from five replications. At each ratio, values followed by the same letter are not significantly different according to Duncan's multiple range test at P = 0.05. A: leaves above the Xoo-inoculated leaf, B: leaves below the Xoo-inoculated leaf.*

4 Discussion

The elicitor of SA effectively induced the resistance of rice plants against *Xoo*, reducing the expansion of LB lesions, under net house conditions. This study is in conjunction with earlier reports in rice and soybean in which exogenous SA elicits the production of resistance materials that lead to biochemical and structural modifications associated with disease resistance [14, 22, 23]. The reduction of disease severity of 1 mM SA at 14 DAI in the study is 41.41%, in accordance to previous research of Le Thanh et al. [14], at approximately 38.17%. Foliar sprays with SA at concentrations of 8 and 10 mM inhibited disease incidence of rice blast at the level of approximately 60-70 % [22]. In addition, application of SA at a concentration of 0.6 mM could enhance pectin and lignin in soybean plants, leading to enhance plant tolerance to drought stress [23]. One main advantage of induced resistance is a systemic characteristic which could elicit, transport and increase the ability of disease resistance in other parts of plants. Some questions remain as what are biochemical changes involved LB resistance could occur at the leaves above and below the *Xoo*-inoculated leaf.

At the above leaves, biochemical modifications are less formed of defence molecules, showing on only one change at the vibrational peak at 1734 cm^{-1} (C=O esters). SA-induced LB resistance could result in alterations of lignin and pectin compositions in rice leaves. The results of this study indicated that SA-treated leaves had higher ratios of 1233/1517, 1467/1517 and 1735/1517 cm^{-1} wavelengths than in the control treatment. The first ratio of 1233/1517 cm^{-1} represents a methoxyphenolic substitution in aromatic units of lignin [24]. Ratio of 1467/1517 cm^{-1} is the ratio of syringyl monomer to guaiacyl monomer of lignin molecules [25]. Besides, ratio of 1735/1517 cm-1 is representative of an alteration in pectin [26]. In general, the facts of significant lignin and pectin alterations in leaf cells in rice, not an accumulation of proteins and lipids, are responsible to a formation of biochemical barriers of disease resistance.

Meanwhile, salicylic application in rice seed and foliage resulted in increased amounts of defensive carbohydrates, lipids and proteins at the below leaves. SA treatment promoted significantly higher spectra shifts at several vibrational peaks, including 2920, 2851, 1736, 1103 and 1040 cm^{-1}, all assigned to defence molecules of carbohydrates and lipids. Some authors proved that carbohydrates in plant cells play a key role in plant - pathogen interactions, leading to other various plant defence responses [27]. In addition, plant lipids contribute to a production of SA and an induction of systemic resistance [28]. Another possible defence molecule of SA effect on induced resistance lies in the fact that alpha helix structure of the amide I protein in SA-treated leaves was changed to beta sheet structure. The latter structure of the amide I protein was suggested to uncover its high affinity binding site for rice receptors, leading to create stronger and faster systemic defence signals against further pathogen invasions [29, 30].

5 Conclusion

The seed and foliar application of SA at a concentration of 1 mM significantly decreased the severity index of LB disease in rice cv. KDML 105. The main finding of this study was to characterize biochemical defence activities in above and below leaves, strongly confirmed the actual systemic mechanism of induced resistance in rice plants. Between these two facts, complementary research should address the efficacy of elicitor of SA in field experiments to fully comprehend the role of SA in rice LB resistance.

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