International Journal of Agriculture, Environment and Bioresearch

Vol. 5, No. 04; 2020

ISSN: 2456-8643

COMPARISION OF BIOCHEMICAL CHARACTERISTICS ON INDUCED RESISTANCE AGAINST LEAF BLIGHT DISEASE IN RICE

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https://doi.org/10.35410/IJAEB.2020.5540

ABSTRACT

The aim of this study was to compare biochemical traits of two rice leaf positions in induced resistance by 1,2-Benzisothiazol-3 (2H)-one 1,1-dioxide (BIT) at a concentration of 2 mM against leaf blight in rice plants. The results showed that BIT-induced rice leaves had many intense peaks which represented defensive carbohydrates, proteins and lipids. At leaves above the inoculated leaf, exogenous BIT treatment had higher peaks of lipids and proteins, such as 2920, 2851, 1736 cm-1, and the structural change of amide I from alpha helix type at the peak of 1655 cm-1 to β-sheet type at 1636 cm-1. At rice leaves below the inoculated leaf, its Fourier transform infrared peak assignments of the BIT-induced treatment had significantly spectral peaks at some vibrational peaks of lipids and carbohydrates, such as 2920, 2851, 1319, 1103 and 1040 cm-1. In this study, the elicitor of BIT reduced leaf blight severity by approximately 34.82%.

Keywords: bacterial leaf blight, elicitor, induced resistance, rice.

1. INTRODUCTION

Rice (*Oryza sativa* L.) is the major food at the world, providing food safety and livelihoods for billions of people [1] [2]. Rice plays an important role on nutrition and health implication [3]. Rice leaf blight (LB) disease caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) was the most frequently disease at fields in Asia and West Africa [4] [5]. In India, Indonesia, Japan and the Philippines, LB damage was reported to a range of 20-30% and can reach 75%, depending on locations, rice varieties and environmental conditions [6] [7] [8]. In Africa countries, yield losses caused by *Xoo* was estimated approximately 30-50% [9]. The severity of LB losses required the development of eco-friendly and cost-effective strategies for managing. Management of LB is mainly focused on methods of using agro-chemicals, resistant varieties and systemic acquired resistance (SAR) to reduce the initial inoculum and enhance plant health [10] [11]. The SAR,

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based on the increased expression of genes in the host plants, could elicit natural defense mechanisms in rice. The induced plants is able to resist an attack of virulent pathogens by enhancing an array of rapidly expressed defenses upon infection [12]. Induction of disease resistance could be resulted from biotic and abiotic elicitors [13] [14] [15]. In *Arabidopsis*, foliar sprays of 2 mM 1,2-Benzisothiazol-3 (2H)-one 1,1-dioxide (BIT), could induce an accumulation of salicylic acid (SA) by stimulating the SAR pathway. Levels of free and total SA in the BIT-treated *Arabidopsis* were approximately 7- and 5-fold greater, respectively, at 5 days after treatment [16]. The treatments of 10 and 20 mM salicylic acid, 0.5 and 2 mM jasmonic acid had played a role in reducing bacterial disease caused by *Acidovorax avenae* in creeping bentgrass (*Agrostis stolonifera*) [17]. Saccharin could induce SAR responses of soybean (*Glycine max*) against the infection of rust fungus *Phakopsora pachyrhizi* [18]. Similarly, saccharin at a spraying concentration of 0.24 mg mL^{-1} could protect bean plants (*Phaseolus vulgaris*) against rust and angular leaf spot [19]. An elicitor of acibenzolar-S-methyl highly controlled tobacco blue mould [20], could reduce basil downy mildew at approximately 47-94% in disease severity [21].

Protection of rice plants from a bacteria's initial penetration is achieved via passive defenses, such as physical and/or chemical barriers such as plant cuticle, cell walls, phenolic compounds, quinones, tannins. When the bacterial pathogens can pass these host passive barriers, the rice plants continue to generate secondary active defenses, including cell wall appositions, callose deposits, lignification, phytoalexins, hypersensitive response, and pathogenesis-related proteins [4]. These active defenses in induced rice plants happen faster than in non-induced ones.

Fourier transform infrared (FTIR) spectroscopy is a fingerprinting tool to differentiate plant metabolic status. The FTIR technique can be used with several different approaches such as hierarchical cluster analysis, principal component analysis and genetic algorithms to detect spectra and formulate possible explanation based on the metabolic differences among elicitor-treated and control treatments [22] [23].

The aim of this study was to compare alterations in cell walls of two rice leaf positions occurred during the process of BIT-induced resistance against leaf blight disease.

2. MATERIALS AND METHODS

The experiments were carried out at the laboratory and net house of Institute of Agricultural Technology, Suranaree University of Technology and the IR-laboratory of Synchrotron Light Research Institute, Thailand.

2.1 Rice variety and resistance elicitor

Seeds of a susceptible rice variety, KDML105, was supported by Rice Research Center, Thailand. Resistance elicitor of BIT, a metabolite of probenazole, was gifted by Prof. Hideo Nakashita, Fukui Prefectural University, Japan.

2.2 Bacterial strain and culture condition

Aggressive strain of *Xoo* was obtained from Plant Pathology and Biopesticide Laboratory, Suranaree University of Technology, Thailand. The *Xoo* was cultured into 300 ml of nutrient

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broth at 27±2°C for 72 h, with 180 rpm shaking. Finally, the culture medium was re-suspended in sterile distilled water (DW). The density of the bacterial suspension was determined at 10^8 cfu mL^{-1} based on optical density of 0.2 at 600 nm [24].

2.3 Rice cultivation, induction treatment, disease assessment and sample preparation

The experiment was conducted with completely randomized design (CRD), five replicates. Rice seeds cv. KDML105 were surface-disinfected by a treatment with 90% ethanol (v/v) for three min. Next, 50 g of rice seeds were soaked thoroughly with 100 ml of the solution of BIT at a concentration of 2 mM, incubated on wet papers in the dark conditions for one day. Rice seeds treated were planted in 35 cm-plastic pots containing soil, at a net house with a natural light regime (27°C and 70-80% relative humidity). There were two pots per one replication, with two seeds planted per one pot. The rice plants were further treated by foliar sprays with the solution of 2 mM BIT until it ran off, at 15, 30 and 45 days after planting (DAP). On an untreated control, the rice seeds and plants were prepared identically, but DW was used. At each rice pot, tip of six matured leaves of fifty-day-old plants were randomly chosen, cut and dipped into a *Xoo* suspension at a density of $1x10^8$ cfu mL^{-1} [25] [26]. Following the artificial inoculation, the plants were put in an inoculation room at the dark conditions with relative humidity of approximately 95% at 25^0C for one day. The plants were then kept in the net house with a natural light regime.

LB disease scores were assessed at 7, 14 and 21 days after inoculation (DAI), using a disease scale for assessing rice LB under net house conditions [27]. Disease severity (DS) was calculated as DS (%) = [Sum of all numerical ratings / (Total number of leaves graded x Maximum scale)] x 100%. Reduction of DS (RDS) for BIT-treated treatment was calculated using a formula as follows: RDS (%) = [(DS of control – DS of BIT-treatment) / DS of control] x 100%.

Above or below leaf (Figure 1) were sampled at 7 DAI, put into an oven at 60ºC for approximately 3 days, then ground into fine powder by pestles and mortars. Equal weights of powder samples were taken and analyzed by FTIR spectroscopy.

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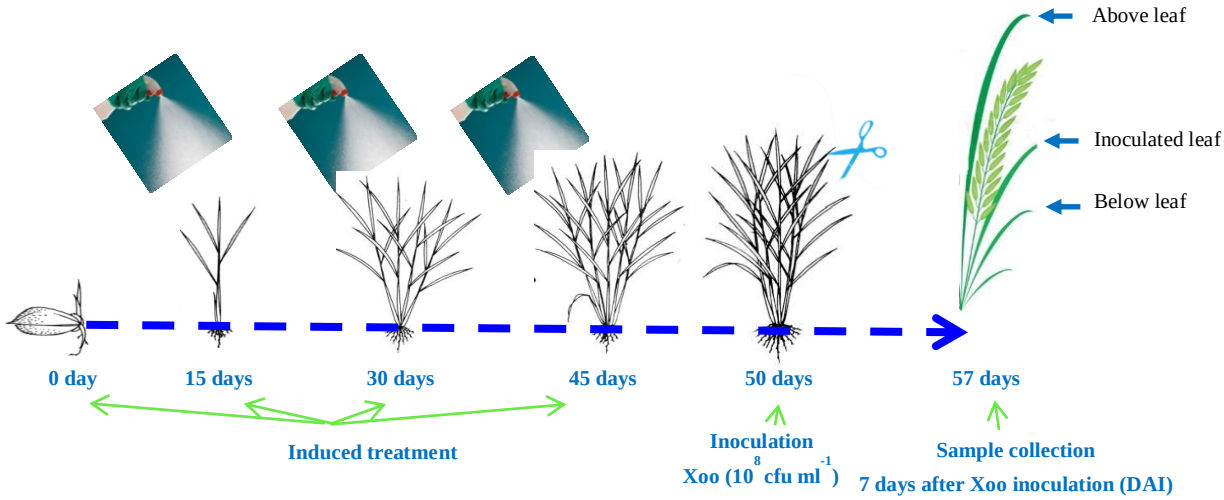

Figure 1: The experimental procedure. Seed soak and foliar sprays with BIT at a concentration of 2mM on rice cv. KDML105 grown under nethouse conditions at 0, 15, 30 and 45 days after planting (DAP). On the untreated control, the rice seeds and plants were handed identically, but distilled water was used instead of BIT. Rice plants were inoculated with *Xanthomonas oryzae* pv. *oryzae* (Xoo) at 50 DAP. Two positions of leaves including above leaf and below leaf were collected at 7 days after Xoo-inoculation.

2.4 Analysis of FTIR spectra

The spectra were measured by using FTIR spectroscope (Bruker Optics Ltd., Ettlingen, Germany). FTIR spectroscopy in the mid infrared (IR) region of 4000-900 cm^{-1} at a spectral resolution of 4 cm^{-1}, with 18 spectra per one sample, at Synchrotron Light Research Institute, Thailand [28] [29].

The analytical procedure consisted in calculating the differences in the IR spectra by using OPUS 6.5 software (Bruker optic, German). The individual spectrum from each group was converted to the second derivative, employing nine smoothing points by Savitzky-Golay method and vector normalized by the Extended Multiplicative Signal Correction, to normalize effect of different thickness of the rice leaf powder samples. Unsupervised Hierarchical Cluster Analysis was carried out on IR data using Ward's algorithm to characterize the various biochemical components of the rice leaf tissues over spectral ranges of 3000-2800 cm^{-1} and 1800-900 cm^{-1}. Peak positions were determined using second derivation [28] [30].

2.5 Statistical analysis

Treatment means were separated by Duncan's Multiple Range Test using SPSS software, version 16 (SPSS, Chicago, IL). Significance was determined by the magnitude of F-value at $p = 0.05$. All experiments in the research were repeated three times, with similar results in all replicates.

3. RESULTS AND DISCUSSION

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The elicitor BIT at a concentration of 2 mM was assessed for its efficacy on SAR by seed soak and foliar sprays at 15, 30 and 45 DAP. The results indicated that the treatment of 2 mM BIT importantly decreased the DS of LB at three observing time points, confirming that the efficacy of BIT on SAR was happened. The DS of BIT-treated rice plants were approximately 19.52, 27.62 and 34.76% at 7, 14 and 21 DAI, respectively, significantly lower than those of the control one which were 27.14, 37.62 and 53.33%. Reduction of DS in the treatment of 2 mM BIT was approximately 34.82% at 21 DAI (Table 1).

Table 1: Efficacy of BIT at a concentration of 2mM on severity and reduction of BLB disease in rice cv. KDML 105 caused by *Xanthomonas oryzae* pv. *oryzae* under net house conditions

Treatment	Disease severity[1/] (%)			Reduction of disease severity compared with control (%)		
	7 DAI [2/]	14 DAI [2/]	21 DAI [2/]	7 DAI [2/]	14 DAI [2/]	21 DAI [2/]
BIT-treated	19.52±3.91b	27.62±2.13b	34.76±2.12b	28.08	26.58	34.82
Non-treated control	27.14±2.71a	37.62±6.16a	53.33±5.73a			
Significance	**	**	**			
Coefficient of Variation (%)	5.12	6.83	10.60			

Rice plants were treated by seed soak and foliar spays at 15, 30 and 45 days after planting (DAP), with 2 mM BIT or distilled water as a control, and then inoculated with *Xanthomonas oryzae* pv. *oryzae* suspension at a density of $1x10^8$ cfu ml^{-1} at 50 DAS. The data were means ± S.E. with five replications, two rice plants per one replication, three leaves per one rice plant. BIT-treated: susceptible rice cultivar cv. KDML105 treated with BIT at a concentration of 2 mM; Non-treated control: susceptible rice cultivar cv. KDML105 treated with distilled water.

[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

In order to compare the effect of elicitor BIT on the systemic defense responses of two rice leaf positions, the biochemical alterations of rice leaves were characterized by FTIR spectroscopy analysis. Original and second derivative average spectra of rice leaves of BIT-induced treatment and diseased control in the range of 3000-2800 and 1800-900 cm^{-1}, were shown in Fig. 2 and 3.

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Biochemical roles of specific spectral peaks were described in several papers of bio-researches, but the study of FTIR on induced resistance is not much (Table 2). The spectral range of 3000-2800 cm^{-1} was shown in Fig. 3a and 3c. The broad bands at the peaks of 2920 and 2851 cm^{-1}, both belong to C–H asymmetric and symmetric vibration, were more intense in treatment of BIT. The differences on spectral ranges of 1800-900 cm^{-1} of non-treated and BIT-treated leaves are shown in Fig. 3b and 3d. At above rice leaves, the BIT-treated treatment had one importantly higher peak at 1736 cm^{-1}, assigned to stretching vibration of C=O ester. Moreover, alpha helix form (1655 cm^{-1}) of amide I in BIT-treated rice leaves appeared more intense than β-sheet one (1636 cm^{-1}). However, the alpha helix peak greatly remained in the control (Fig. 3b). At below rice leaves, their FTIR peak assignments of the BIT-induced treatment had three importantly higher vibrational peaks, including 1319 cm^{-1} (assigned to C-C, C-O skeletal of hemicellulose and lignin), 1103 cm^{-1} (C-O-C glycoside of hemicellulose) and 1040 cm^{-1} (C-O-C of polysaccharides) (Fig. 3d). One of the most important characteristics of induced resistance is systemic. After the first infection from *Xoo*, induced rice plants could create signals and defense responses at other parts of plants, both above and below rice leaves. The interesting questions remain to be explored are which leaf position has more biochemical defense responses in and what defense responses at each leaf position. The results showed that above leaves have increased defense responses on lipids and protein, while below leaves have more significant alterations on lipids and carbohydrates.

Table 2: Band assignments of FTIR vibration peak (cm^{-1}) of plant rice leaf tissues based on references

Peak name	Spectral ranges	Vibration peak assignments	References
C-H stretching vibration	3000-2800	C-H Asymmetric and Symmetric stretching vibration of mainly lipid groups with the little contribution from protein,	[31] [32] [33]
C=O esters	1740-1700	Stretching vibration of C=O ester of bond lipid, lignin, pectin or their esters	[31] [33] [34] [35] [36]
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%))	[33] [34] [37] [38]
Amide II	1600-1500	Amide II due to N-H bending and C-N stretching of protein (N-H bend (60%), C-N	[34]

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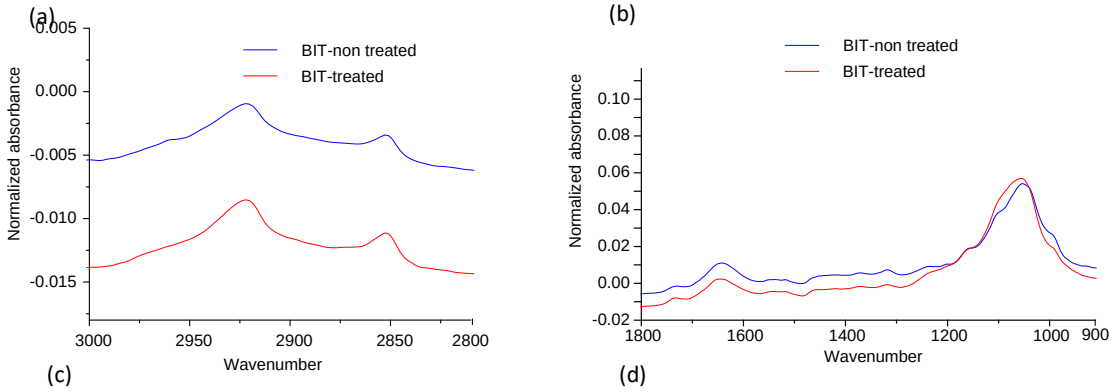
Peak name	Spectral ranges	Vibration peak assignments	References
		stretch (40%))	
C=O aromatic ring	1517	C=C aromatic ring from lignin, C-H bend	[32] [33] [34] [39]
C-H bending	1470-1350	C-H bending from CH2 and CH3 from mainly lipids and lignin	[34] [35]
C-O stretching hemicellulose and lignin	1300-1200	C-C, C-O skeletal	[32] [40]
C-C ring cellulose	1165	C-C ring from cellulose	[34] [40] [41]
C-O-C glycoside	1103	C-O-C glycoside ether mainly hemicellulose	[36]
C-C bond of cellulose	1080-1022	Stretching vibration of C-OH of alcohic groups and carboxylic acid, C-C bond of the cellulose sugar rings. Mainly C-O-C of polysaccharides	[31] [34] [40] [41]
C-O bonds of sucrose	995-988	endocyclic and exocyclic C-O bonds of cello–triose, –tetraose, and –pentose	[40] [42]



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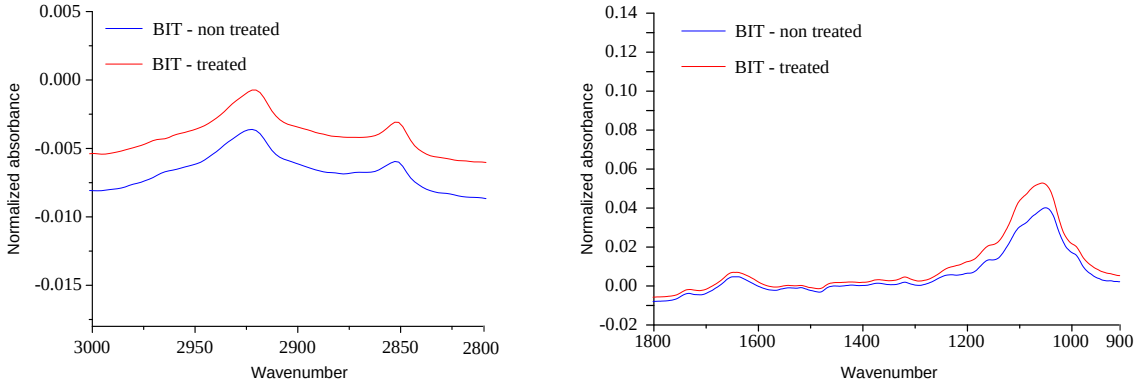

Figure 2: Representative original average FTIR spectra in KDML 105 rice leaves treated with 2 mM BIT and inoculated with *Xoo*, at 7 DAI, under nethouse conditions. (a), (b) Representative original average FTIR spectra in leaves above the inoculated leaf; (c), (d) Representative original average FTIR spectra in leaves below the inoculated leaf. Twelve spectra per group were preprocessed by taken second derivative spectra after 9 points of smoothing and normalized with EMSC over the range.

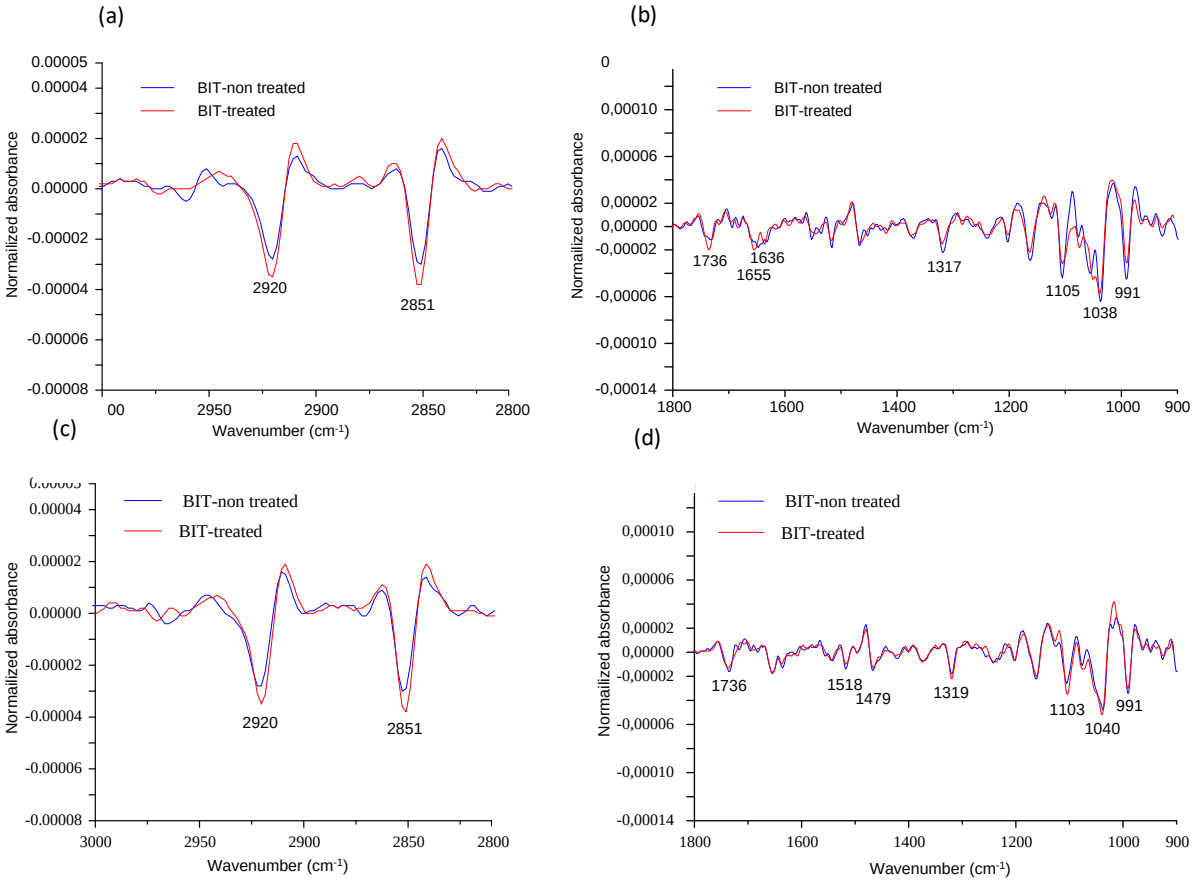

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Figure 3: Second derivative average spectra in KDML 105 rice leaves treated with 2 mM BIT and inoculated with *Xoo*, at 7 DAI, under nethouse conditions. (a), (b) Second derivative average spectra in leaves above the inoculated leaf; (c), (d) Second derivative average spectra in leaves below the inoculated leaf. Twelve spectra per group were preprocessed by taken second derivative spectra after 9 points of smoothing and normalized with EMSC over the range.

Principal component analysis (PCA) is a common technique for a reduction of sample dimensionality. Two-dimensional PCA analyses in above and below rice leaves were presented on Fig. 4 and 5, respectively. The blue points of the control were separated from the red points representing the BIT-treatment, both in above (Fig. 4a) and below (Fig. 5a) rice leaves

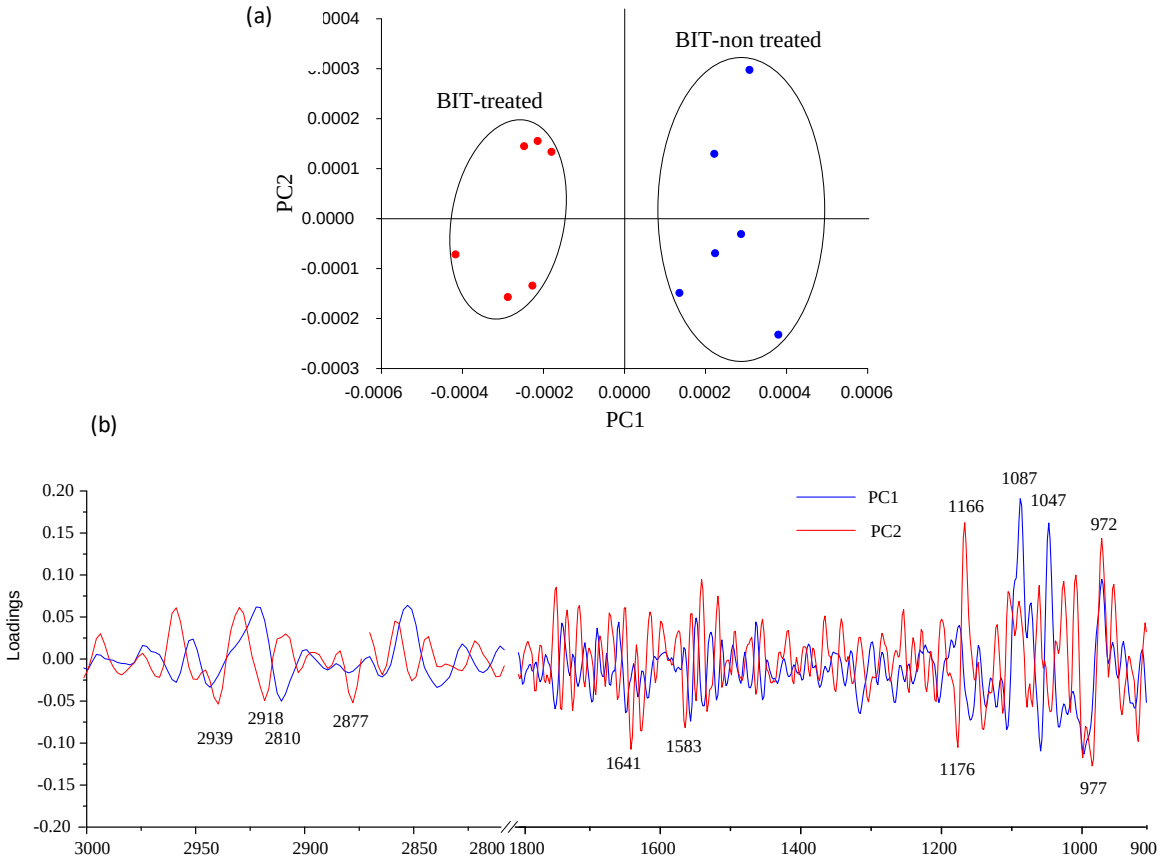

Figure 4: PCA analysis in rice leaves above the inoculated leaf, at 7 DAI, under nethouse conditions. (a) 2D scatter plot of score from a PCA analysis. (b) Loading plots from a PCA analysis in the range of 3000-2800 and 1800-900 cm^{-1}

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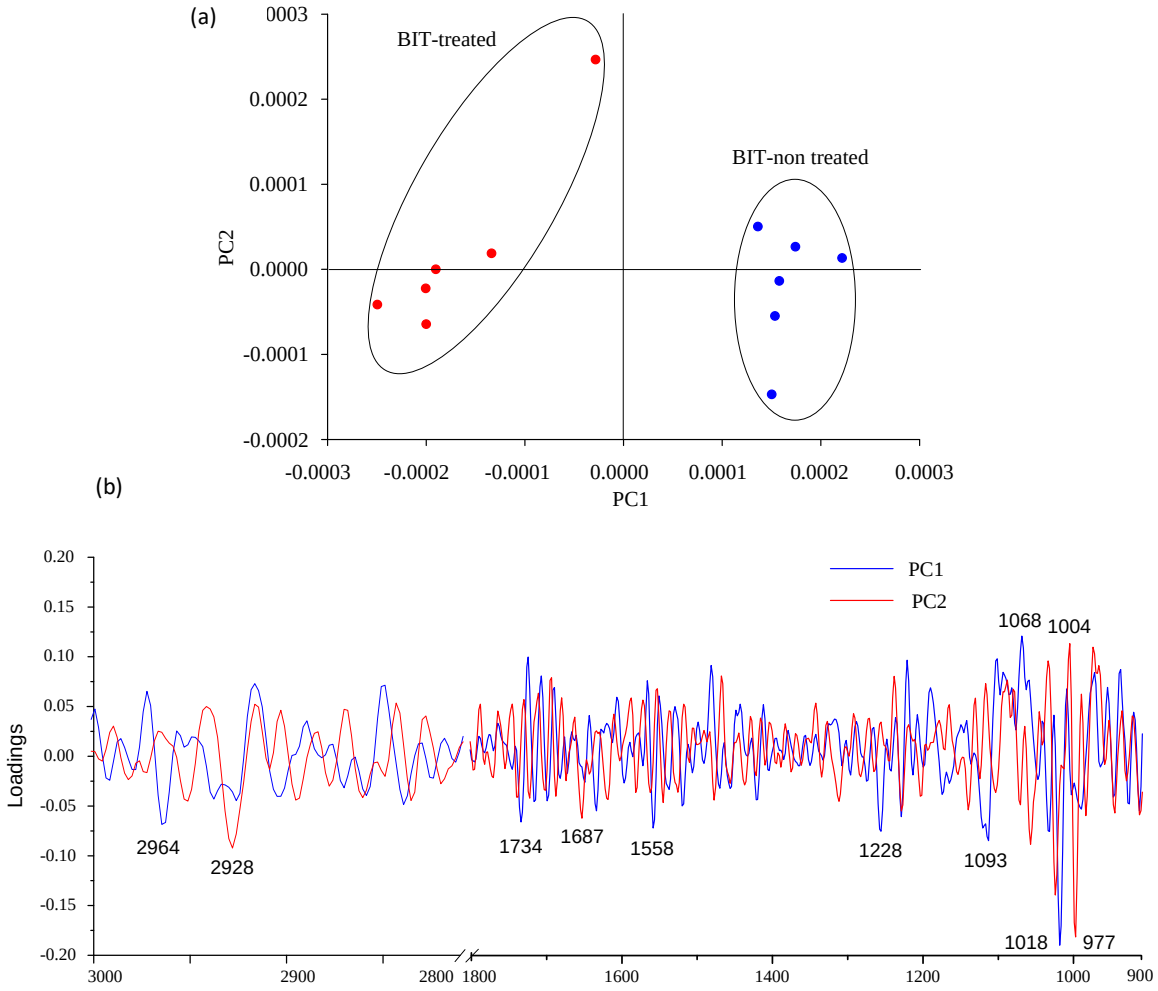

Figure 5: PCA analysis in rice leaves below the inoculated leaf, at 7 DAI, under nethouse conditions. (a) 2D scatter plot of score from a PCA analysis. (b) Loading plots from a PCA analysis in the range of 3000-2800 and 1800-900 cm^{-1}.

A classification procedure by the cluster analysis was carried out to investigate more information about the biochemical differences among the cell wall of rice leaves on induced resistance (Fig. 6). The cluster analysis is a technique to examine inter-point distances between rice samples as well as presents that information in the type of a two-dimensional plot or a dendrogram. The dendrogram of above leaves' spectra displays two main branches which separated approximately 0.6 unit. The spectra within the above branch are the treatment treated by BIT at a concentration of 2 mM. The below branch contains only the diseased control (Fig. 6a). Lastly, the dendrogram corresponding to the cell wall spectra from below leaves is displayed in Fig. 6c. At 7 DAI, the above branch is separated by 0.4-0.6 unit from the below one and comprised of all spectra of the BIT-treated and disease control, respectively.

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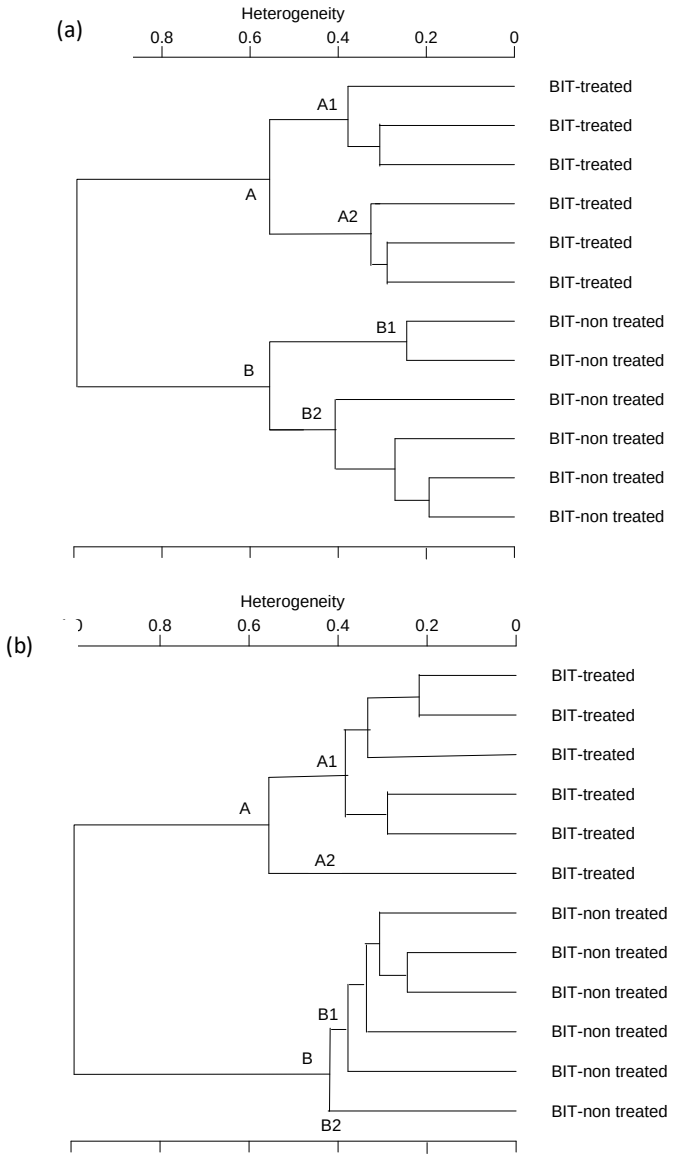

Figure 6: Cluster analysis of FTIR spectra in the range of 3000-2800 and 1800-900 cm^{-1} in rice leaves, at 7 DAI, under nethouse conditions**.** (a): leaves above the inoculated leaf, (b): leaves below the inoculated leaf

In addition, to further find out alterations of lignins and pectins in BIT-treated rice leaves, three ratios of 1233/1517, 1467/1517, and 1735/1517 cm^{-1} were specified. At above and below leaves, these biochemical ratios of BIT-treated treatment showed that approximately 20-80 percentage of

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pectins and lignins were significantly accumulated (Fig. 7a and 7b).

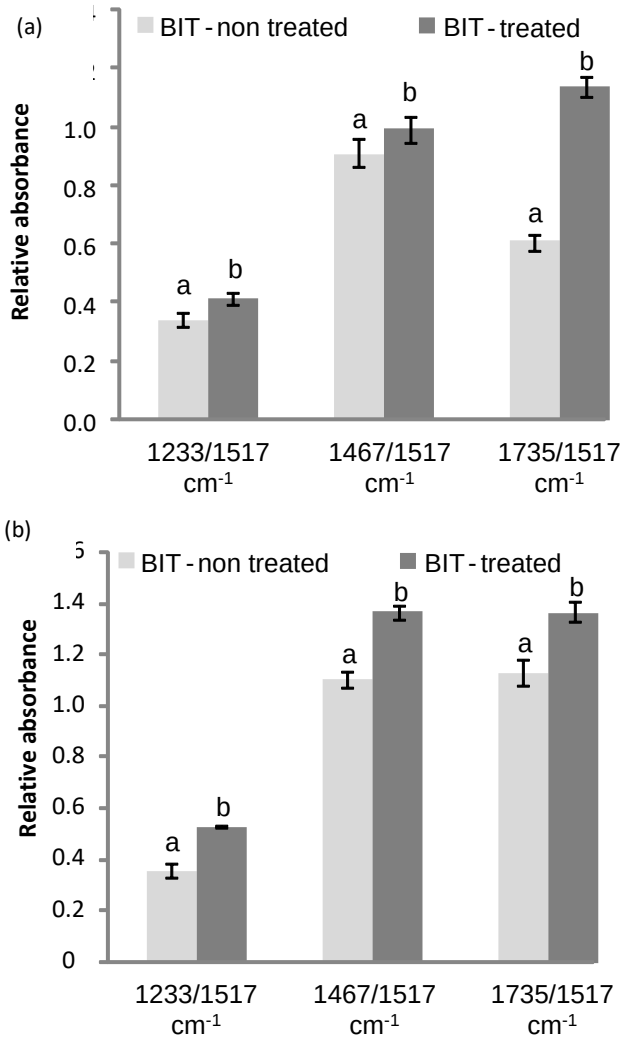

Figure 7: Relative absorbance ratio of some spectral peaks to the intensitive at 1517 cm^{-1} in KDML 105 rice leaves treated or non treated with BIT 2mM, at 7 DAI, under nethouse conditions. Error bars represent standard deviation from 6 replications. At each ratio assessed, values followed by the same 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. (a): leaves above the inoculated leaf, (b): leaves below the inoculated rice leaf

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Many available analysis tools can be used for studying induced resistance at cellular and subcellular levels as plant histopathological methods, transcriptome, proteome, secondary metabolites [43] [44]. Cell wall components can be analyzed by many methods, including immunolocalization, GC-MS and histochemical staining [45] [46]. However, these methods require labor-intensive in case of treating lots of samples [39]. FTIR is advantageous because the strategy to reveal good spectra of each biological sample. FTIR combined with several different statistical approaches could be a valuable method to detect, classify and formulate the contribution of biological components on the metabolic differences among treated and control treatments [22] [32]. However, few studies were carried on using FTIR as a tool to identify changes in cellular components on induced resistance, especially in the rice plant.

Resistance to LB disease in rice leaves largely resulted from the ability of their cells to modify the composition and structure of their cell walls. The below rice leaves showed more biochemical alterations in the cell walls than above ones. Therefore, below leaves should be used for the identification of cell wall changes related to assess the ability of the elicitor on induced resistance against pathogens in rice plants. On qualitative analyses, FTIR absorption band area values of C-C, C-O skeletal of hemicellulose and lignin, C-O-C glycoside of hemicellulose, and C-O-C of polysaccharides could be used as indicators of induced resistance to LB disease in rice plants. Wang *et al.* [39] investigated the composition of wheat cell wall contributing to stem lodging resistance by FTIR spectroscopy and concluded that it was not possible to use only one peak intensity as a predictor on FTIR spectra between different developmental internodes.

4. CONCLUSION

The currently available information on induced resistance in this study suggests that the elicitor of BIT has a great potential application on LB management. FTIR spectroscopy offers a new tool to characterize biochemical alterations on rice plant responses to LB disease. Emphasis has given to the changes of defensive carbohydrates, proteins and lipids in induced rice plants. The above rice leaves have dominant defense responses on lipids and proteins, while activities of resistance lipids and carbohydrates occur highly on the below rice leaves. The precious information of spectral peaks such as C-C, C-O skeletal of hemicellulose and lignin, C-O-C glycoside of hemicellulose, and C-O-C of polysaccharides is also used as biomarkers to quickly investigate the occurrence of disease resistance in induced rice plants.

ACKNOWLEDGEMENTS

Authors wish to express our special thanks to the SUT-ASEAN scholarship from Suranaree University of Technology for providing the financial support.

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