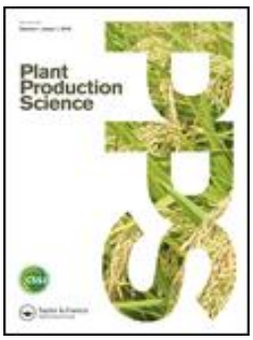

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Metabolite profiling of sheath blight disease resistance in rice: in the case of positive ion mode analysis by CE/TOF-MS

Woro Sri Suharti[a,b], Akihiro Nose[a] and Shao-Hui Zheng[a]

[a]Faculty of Agriculture, Saga University, Saga, Japan; [b]Faculty of Agriculture, Jenderal Soedirman University, Purwokerto, Indonesia

ABSTRACT

Rice sheath blight is an important disease caused by *Rhizoctonia solani*. The resistant and susceptible rice lines (32R and 29S, respectively) showed different responses to *R. solani* infection in metabolite levels. The aim of this study was to characterize the metabolite levels in rice lines during *R. solani* infection using capillary electrophoresis equipped with time of flight mass spectrophotometry (CE/TOF-MS) in positive ion mode. Hundred metabolites were identified and classified into six clusters by hierarchical cluster using Mass Profiler Professional software. Changes in metabolite level at inoculated 32R and 29S were mapped on branches of tricarboxylic acid and glycolysis pathway. Volcano plot successfully filtered the metabolites based on fold change and *p*-value. The volcano plot result showed that 10 metabolites were up and down regulated in inoculated 32R relative to 29S. One metabolite, chlorogenic acid, showed a positive response in 32R. Meanwhile, pipecolic acid showed as the highest magnitude of fold change and *p*-value significance level in 29S. In addition, eight amino acids; glutamate, γ-aminobutyric acid, glycine, histidine, phenylalanine, serine, tryptophan, and tyrosine showed increase in 29S after *R. solani* inoculation.

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KEYWORDS
Amino acid; CE/TOF-MS; phenol; resistance; rice line; *Rhizoctonia solani*

Introduction

Rice sheath blight disease, caused by *Rhizoctonia solani* is a major disease affecting rice cultivation. The disease decreases the rice yield by 50% in the field (Lee & Rush, 1983). Cultural practices and biological and chemical controls have been developed to ameliorate the problem (Nagarajkumar et al., 2004; Rodrigues et al., 2003; Slaton et al., 2003). Several breeding techniques, such as variety screening and transgenic biotechnologies, have been introduced to obtain resistant varieties (Datta et al., 1999; Jia et al., 2006).

According to Wasano and Dhanapala (1982), there are two types of plant disease resistance that mediated the interaction between plant and pathogen, major gene and polygene. Nevertheless, Li et al. (1995) noted that the major gene resistance to the *R. solani* have not been identified. Therefore, the inheritance of rice resistance to sheath blight disease is mostly controlled by polygene. Polygene resistant is more desirable because of its durability (Parlevliet 2002). Wasano et al. (1985) identified $2F_{18}$-7-32 (32R, resistant line) and $2F_{21}$-21-29 (29S, susceptible line) as polygene resistance lines by crossbreeding derived from Tetep × CN4-4-2. Tetep is known as a resistant *Indica* variety to rice sheath blight disease. Meanwhile, CN4-4-2 is susceptible hybrid *Japonica* rice from Chugoku 45 and Nipponbare (Wasano & Hirota, 1986). These resistant (32R) and susceptible (29S) rice lines were considered to be useful for the analysis of the mechanism of resistance to the sheath blight disease in rice.

Previous studies in our laboratory (Danson et al., 2000; Mutuku & Nose, 2010, 2012) indicated that there were different physiological responses in 32R and 29S rice lines after infection of R. solani. Several key enzymes and metabolites in the phenylpropanoid and shikimate pathways are increased after R. solani infection (Mutuku & Nose, 2012). Phenylpropanoid and shikimate pathways are involved in plant defence mechanism (Dixon et al., 2002; Tzin & Galili, 2010) and are responsible for the synthesis of secondary metabolites, including phenol (Lattanzio et al., 2006). It has been suggested that biosynthesis of secondary metabolites, such as phenols, plays an important role in plant resistance to R. solani infection (Akhtar et al., 2011).

Previous studies have also shown that the mRNA expression of phenylalanine ammonia lyase (PAL) enzyme in the phenylpropanoid pathway increases in 32R after infection of *R. solani* (Mutuku & Nose, 2010). PAL functions is a key step in the biosynthesis of phenylpropanoids in assisting the deamination of the phenylalanine (Dixon et al., 2002), including lignin (Douglas, 1996). Lignin deposition is one of the defence mechanisms used in the response of

CONTACT Akihiro Nose nosea@cc.saga-u.ac.jp

host plants to pathogen infection (Vance, 1980). Danson (1999) indicated that lignin deposition was observed in the sheath and the flag leaf of 32R rice lines after *R. solani* infection.

The levels of several metabolites in 32R and 29S rice lines changed after *R. solani* infection (Mutuku & Nose, 2012). It has also been reported that the level of amino acids change during pathogen infection (Aliferis & Jabaji, 2012). Marked changes in the levels of certain amino acids associated with the changes in gene expression and enzymatic reactions (Rojas et al., 2014).

Mutuku and Nose (2010, 2012) showed a physiological effect of biotic stress on the rice plant shown by enzyme activation, metabolites changes, and lignin accumulation in infection site. Study by Danson (1999) showed the genetical effect on the rice plant during biotic stress as indicated by the presence of enzymatic reactions and accumulation of lignin on the leaves distant from the inoculation site. In this study, capillary electrophoresis/time of flight-mass spectrometry (CE/TOF-MS) analysis was used to improve the understanding of multiple expression of the polygenes that relating to resistant and susceptible rice line. Furthermore, the research by using CE/TOF-MS in positive mode was used to determine the amino acid and phenol compound in rice leaves due to *R. solani* infection.

Material and methods

Sample preparation

The resistant rice line, 32R, and susceptible rice line, 29S, were used in this study. The rice lines derived from Tetep (*Indica*) and CN4-4-2 (*Japonica*). Tetep is a high resistant variety of rice originating in Vietnam, whereas CN4-4-2 is a progeny of cross between Chugoku 45 and Nipponbare (Wasano et al., 1985). Rice seed was germinated and then transplanted in soil containing peat moss and vermiculite (1:1, v/v). The plants were maintained in a growth chamber (KG-50HLA, Koito Co Ltd., Japan) (16 h light, 8 h dark, 30 °C in light period, 25 °C in a dark period, 70% RH, and 400–420 µmol m^{-2} s^{-1} of photosynthetic photon flux density at the plant level). At the seven leaf growth stage, the plants were inoculated with the pathogen mycelium and transferred to a 28 °C incubator (NK System Biotron, Japan).

Pathogen inoculation and sample collection

The *R. solani* AG-1 isolate C–154, No. 305229 from the Agricultural Resource Gene Bank, Tsukuba, Japan was used for this research. *R. solani* pathogen was cultured in potato sucrose agar (PSA) medium for four days at 28 °C. The mycelium of *R. solani* in PSA was chopped well. Syringe was used to support the inoculation. Inoculation was done by injecting the chopped mycelium using syringe to the interstices between the second and third leaf sheaths from the flag leaf. The inoculation method was conducted in accordance with Wasano et al. (1983). As a control, mock inoculation was done by injecting distilled water at the same part of the different plant. Samples were taken from 10 plants at 10:00 am. The samples were collected and combined from upper most developed leaf, second upper leaf, and third upper leaf. Leaf samples were collected together at one, two, and four days post-inoculation (dpi). The leaf samples were rapidly frozen in liquid nitrogen, crushed to a powder using a chilled mortar and pestle and then stored in liquid nitrogen until use.

Determination of soluble protein concentration using a spectrophotometer

The concentration of soluble protein was determined by the method of Bradford (1976) using a bovine serum albumin as the standard for the assay. Leaf powder (200 mg) was added to 50 mM Tris-HCl buffer at pH 7.9 (2 mL) and homogenized. The material was filtered by one layer of Miracloth (Calbiochem, USA) and vortexes (Genie2 Scientific Industries, USA) for 30 s. The sample was centrifuged at 16,000 g for 20 min at 4 °C using Tomy MX 105 (TOMY Digital Biology Co. Ltd., Japan). The precipitate was re-extracted twice with the same buffer. Soluble protein was measured using a spectrophotometer (UV-1800 Shimadzu, Japan) at 595 nm with BioRad protein assay reagent (BioRad Laboratories, USA).

Determination of free amino acid using a spectrophotometer

The free amino acid was measured using Ninhydrin assay (Yemm et al., 1955) with some modifications. Leaf powder (200 mg) was added by 2 mL of 800 mL L^{-1} ethanol, centrifuged for 30 min at 13,000 *g*. The sample extract was taken (0.1 mL) and added by ninhydrin reagent consist of 10 mg mL^{-1} ninhydrin in 0.5 M citrate buffer (pH 5.5), 870 mL L^{-1} glycerol, and 0.5 M citrate buffer with ratio of 5:12:2. The samples were heated in boiling water for 10 min and cooling at room temperature. The free amino acid was measured at 570 nm in UV-1800 Shimadzu Spectrophotometer with glycine as a standard.

CE/TOF-MS analysis in positive ion mode

Sample preparation for CE/TOF-MS was carried out as described by Soga et al. (2006) with some modifications. The rice leaf powder (30 mg) was plunged into 500 µL mixture solution; consist of 10 mL methanol and 5 µL internal standard solution 1. The internal standard

solution 1 is produced by *Human Metabolome Technologies* Inc., Tsuruoka, *Japan*. It contains methionine sulfone and champor-10-sulfonic acid for the MS quantification (Takahashi & Washio, 2011). The sample solution was vortexed for 30 s, then flash centrifuged (300 g) at 4 °C. The sample solution was added to deionized water (200 µL), followed with 99% chloroform (500 µL), and subsequently centrifuged at 2,300 g for 5 min at 4 °C. Supernatant (400 µL) was centrifugally filtered through a Millipore 5-kDa cut-off filter at 7,900 g for 2 h at 4 °C. The sample filtrate was lyophilized using a centrifugal evaporator (CVE-200D Eyela, Japan) for 2 h, dissolved in methanol (50 µL) containing 5 µL of the internal standard solution 3 (*Human Metabolome Technologies*, Inc., *Japan*), and then vortexed (30 s) and flash centrifuged. The internal standard 3 contains trimesic acid and 3-hydroxynaphtalene-2, 7-disulfonic acid. It is used for calibration of CE migration time (Takahashi & Washio, 2011). Finally, sample solution (10 µL) was added to the CE vials and injected into a CE/TOF-MS machine in positive mode condition. The standard was prepared by dissolving gallic acid (10 µg μL^{-1}) and chlorogenic acid (1 µg μL^{-1}) (WAKO, Japan) in distilled water. The metabolite concentration was determined based on relative area as a result of Masshunter Qualitative software which equipped

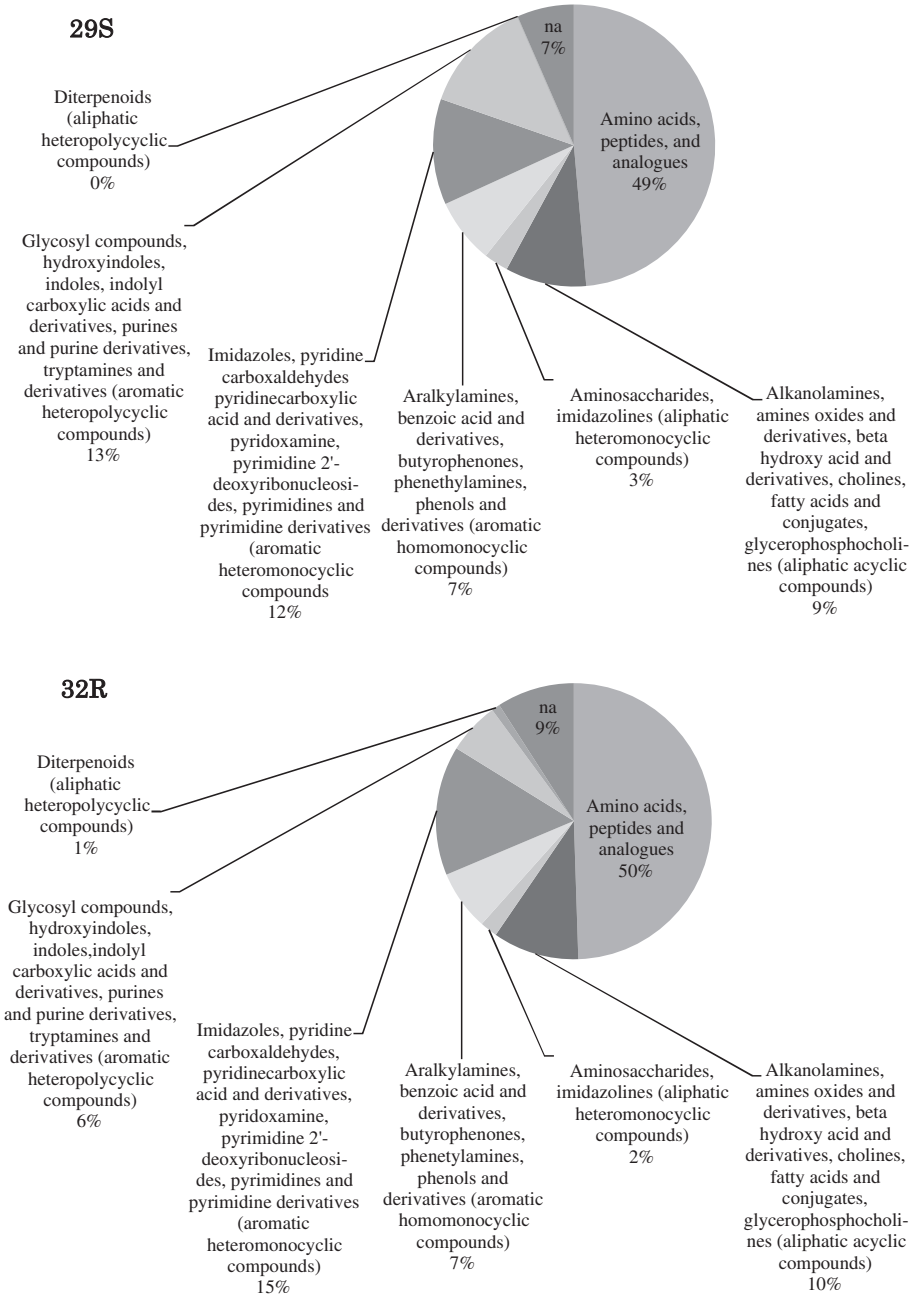

Figure 1. Compound grouping of metabolites in 29S and 32R based on chemical taxonomy.

with annotation by KEGG (*Kyoto Encyclopedia Genes and Genomes*).

CE/TOF-MS Agilent 7100 CE system from Agilent Technologies, USA was used in the experiment in positive ion mode. A fused silica capillary (50 µm i.d. × 80 cm total length) (*Human Metabolome Technologies* Inc., *Japan*) was used for sample separation with buffer solution (*Human Metabolome Technologies* Inc., *Japan*) to provide the electrolyte.

Statistical analysis

Data were analysed by MassHunter (MH) Work Station software on Qualitative Analysis B.05.00 (Agilent Technologies, USA). A Mass Profiler Professional (MPP) B.12.60 (Agilent Technologies, USA) was used to obtain analyse metabolite data. Statistical significance was determined by ANOVA, followed by Tukey test at 5% probability.

Result and discussion

General analysis of CE/TOF-MS on rice lines infected by R. solani

Analysis of the CE/TOF-MS identified 117 metabolites in total. Ninety-nine metabolites were detected in the susceptible line (29S) with mock-inoculated treatment, whereas, 88 metabolites were identified in the resistant line (32R), which also received the same treatment. *R. solani* inoculation decreased the number of detected metabolites in 29S to 90 metabolites. In the inoculated 32R, the number of metabolites increased to 91. Based on chemical taxonomy for the detected metabolites, 49% of 29S and 50% of 32R were identified as members of the amino acids, peptides, and analogues group (Figure 1).

The results of the CE/TOF-MS experiment were further analysed by MPP. MPP analysis software from Agilent is designed to explore the information contained within the mass spectrometry data and perform excellent statistical

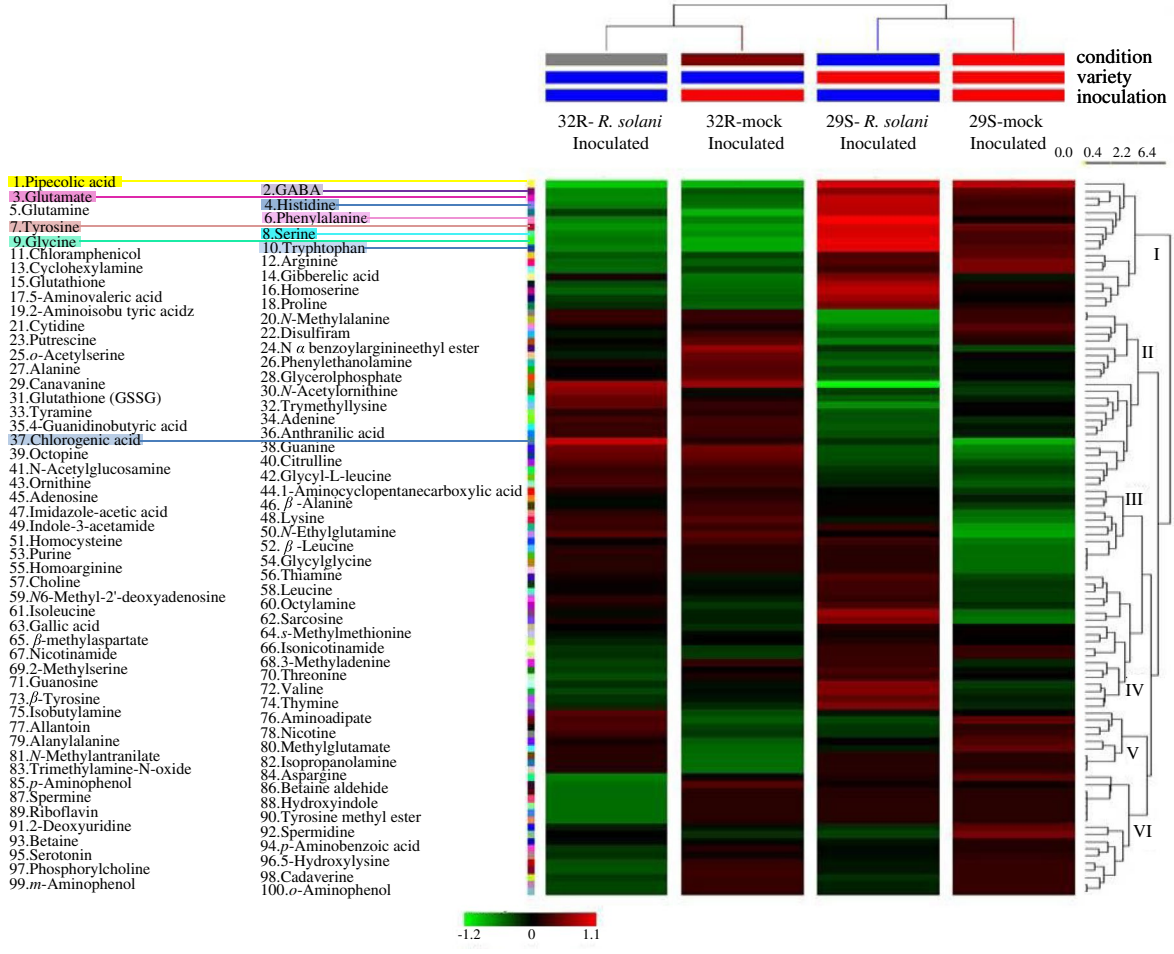

Figure 2. Hierarchical cluster analysis of identified cation metabolite on susceptible (29S) and resistant (32R) rice lines between *R. solani* inoculated and mock control at average of 1dpi, 2dpi, and 4dpi. Chlorogenic acid and gallic acid as phenol standard was added as external standard. Colour intensity related to the degree of increase (red) and decrease (green) of the mean metabolite ratio. The Romans numeral referred to the cluster number.

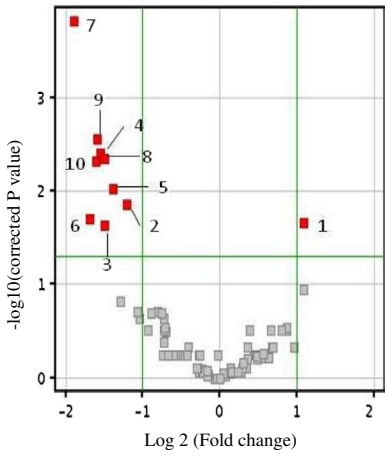

Figure 3. Volcano plot analysis illustrated of identified metabolite between resistant (32R) and susceptible (29S) rice lines after inoculated by *R. solani* at average of 1dpi, 2dpi, and 4dpi. Red square represents the metabolite displayed with larger magnitude fold changes (x-axis, FC ≥ 2.0) and statistical significance difference (y-axis, P ≤ 0.05). Metabolites numbering in volcano plot are accordance with metabolites number as listed in Table 1.

analysis. Each identified metabolite from 32R and 29S treated with mock and *R. solani* was visualized by hierarchical cluster analysis (Figure 2). By using the MPP software, 100 metabolites, including two external phenol standards, were detected in both rice lines. The rice lines with treatment in the columns and metabolites in the rows were displayed.

All metabolites were classified into six clusters at level dissimilarity 2.2 by hierarchical cluster based on Euclidean distance (Figure 2). First cluster showed the group of metabolite in inoculated 29S by the highest level compared with other treatment. A second cluster showed that the metabolite levels of 32R were higher than those of 29S. In the cluster III the level of metabolites in mock inoculated 29S was shown lower than other treatment. Classification in cluster IV displayed that the metabolite group in inoculated 29S was higher than the other. Cluster V and VI showed mock inoculated 29S were higher than other treatment. However, the colour intensity of inoculated 32R and mock inoculated 29S in cluster V were almost similar.

Volcano plot was chosen as an advanced analysis to characterize the role of metabolic response in 32R and 29S after pathogen infection based on fold changes and *p*-value in statistical significance levels (Figure 3). The horizontal and vertical axis show the fold changes (a log scale) and the *p*-values (negative log scale), respectively. Ten metabolites were identified among inoculated 32R and 29S rice line according with a significance threshold of *p*-value ≤0.05 and fold change ≥2.0 (Figure 3 and Table 1). Chlorogenic acid showed a positive fold change in inoculated 32R. Higher levels of pipecolic acid, γ-aminobutyric acid (GABA), glutamate, glycine, histidine, phenylalanine, serine, tryptophan, and tyrosine were detected in inoculated 29S. Based on hierarchical cluster, these nine metabolites were grouped in cluster I, whereas chlorogenic acid was included in cluster II (Figure 2).

The changes in metabolite level in inoculated 32R and 29S by *R. solani* in the metabolic map were shown in Figures 4 and 5. Casting a glance, the distribution of metabolites in the metabolic map was observed as a random spread in the branches of tricarboxylic acid (TCA) and glycolysis. However, chlorogenic acid showed up-regulating, whereas phenylalanine was down-regulating. The chlorogenic acid was derived from phospoenolpyruvate through phenylalanine shown in Figures 4 and 5. In 32R, chlorogenic acid showed a positive response to pathogen infection. Meanwhile, opposite appearance was occurred in the 29S.

Table 1. Up-down regulation metabolite and abundance in volcano plot analysis of resistant (32R) and susceptible (29S) rice lines after inoculated by R. solani.

No. of compound	Compound	Migration Time(min)	*m/z*	FC (abs)	Regulation	FC	Log 2 (FC)	Abund Diff (Raw)	Abund Diff (Log2)
1.	Chlorogenic acid	24.03	355.1099	2.126	Up	2.126	1.088	0.080	−3.650
2.	GABA	9.60	104.0706	2.320	Down	−2.320	−1.214	−1.322	−0.403
3.	Glutamate	10.93	148.0604	2.819	Down	−2.819	−1.495	−5.164	−2.369
4.	Glycine	8.33	76.0393	2.945	Down	−2.945	−1.558	−0.104	3.272
5.	Histidine	7.31	156.0768	2.619	Down	−2.619	−1.389	−0.421	1.247
6.	Phenylalanine	11.09	166.0863	3.220	Down	−3.220	−1.687	−0.832	0.265
7.	Pipecolic acid	10.30	130.0863	3.738	Down	−3.738	−1.902	−3.844	−1.943
8.	Serine	10.01	106.0499	2.835	Down	−2.835	−1.503	−1.090	−0.124
9.	Tryptophan	11.02	205.0972	3.013	Down	−3.013	−1.591	−0.923	0.115
10.	Tyrosine	11.34	182.0812	3.053	Down	−3.053	−1.610	−0.325	1.620

FC; fold change, abs; absolute, Abund diff; abundance differential.

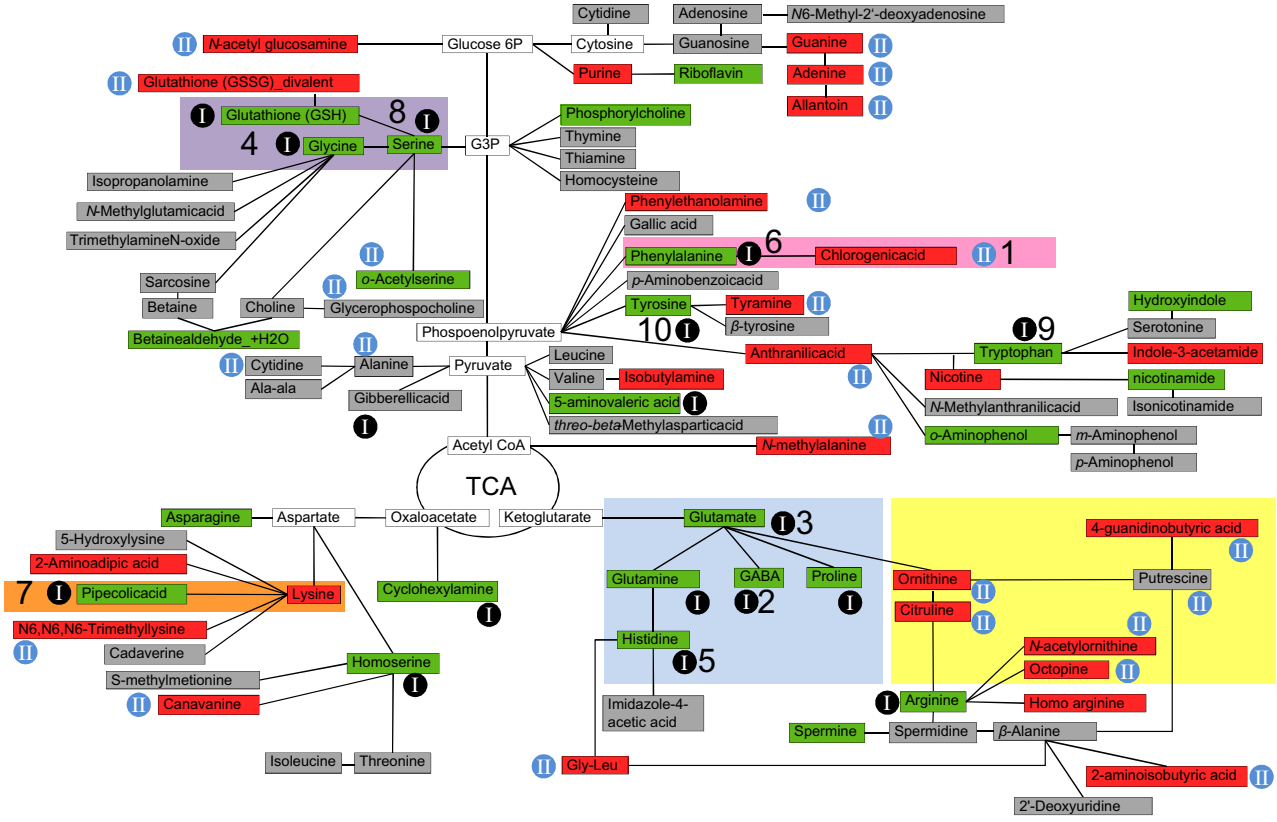

Figure 4. Changes in metabolite levels derived from TCA and glycolysis pathway in 32R inoculated by *R. solani* at average of 1dpi, 2dpi, and 4dpi, based on hierarchical cluster data (Figure 2). Increased and decreased intensity of metabolites were showed in red and green colour. The Romans numeral (I and II) in the figure showed metabolite grouping based on hierarchical cluster. The Arabic number (1 to 10) referred to the number of metabolites related to volcano plot.

As a second property, glutamate was shown as down-regulated metabolite in comparison between inoculated 32R and 29S (Figure 3). Glutamate derivatives were divided into two groups. Glutamine, histidine, GABA, and proline were grouped in cluster I, while ornithine and derivatives were incorporated in cluster II. As shown in Figures 4 and 5, the different flow after glutamate in the metabolic map was observed in inoculated 32R and 29S. In 32R, the level of glutamine, histidine, GABA, and proline decreased. Meanwhile, the level of ornithine and related metabolites increased (Figures 4 and 5). After infection of *R. solani* in 32R, the direction of carbon flow changed to ornithine from glutamate. Meanwhile, in the 29S, the direction of carbon flow altered from glutamate to glutamine, histidine, GABA, and proline after *R. solani* inoculation. Based on the result, it suggested that CE/TOF-MS study can be used as an option to replace the function of stable isotope labelling as carbon flow tracer in the plant. However, Kluger et al. (2014) and You et al. (2014) explained that stable isotope labelling is more effective to trace the metabolite element in cells.

According to the volcano plot analysis, pipecolic acid showed as down-regulation with the highest number of *p*-value and fold change in comparison between inoculated 32R and 29S (Figure 3). Level of pipecolic acid was detected abundantly in inoculated 29S than inoculated 32R (Figures 4 and 5). Pipecolic acid was derived from lysine which detected opposite in the level of metabolite. Probably, susceptible rice plant to *R. solani* was characterized by the existence of pipecolic acid. Detailed discussion on pipecolic acid by using MH Qualitative data is continued in the next section.

Serine, glycine, glutathione (GSH) was derived from glycerate 3 phosphate (G3P) (Figures 4 and 5). Based on hierarchical cluster, these metabolites were included in cluster I. These metabolites showed different responses depending on rice lines. The level of serine, glycine, and GSH in 29S were higher than 32R. The changes of each metabolite level were not shown difference after *R. solani* inoculation. Glycine and serine were down-regulated metabolite showing a significant difference after filtering by *p*-value and fold change (Figure 2).

According to Kushalappa and Gunnaiah (2013), the resistance characteristic in plant against biotic stress can be explained through metabolites mapping in metabolomic approach by metabolic flux observation. The specific

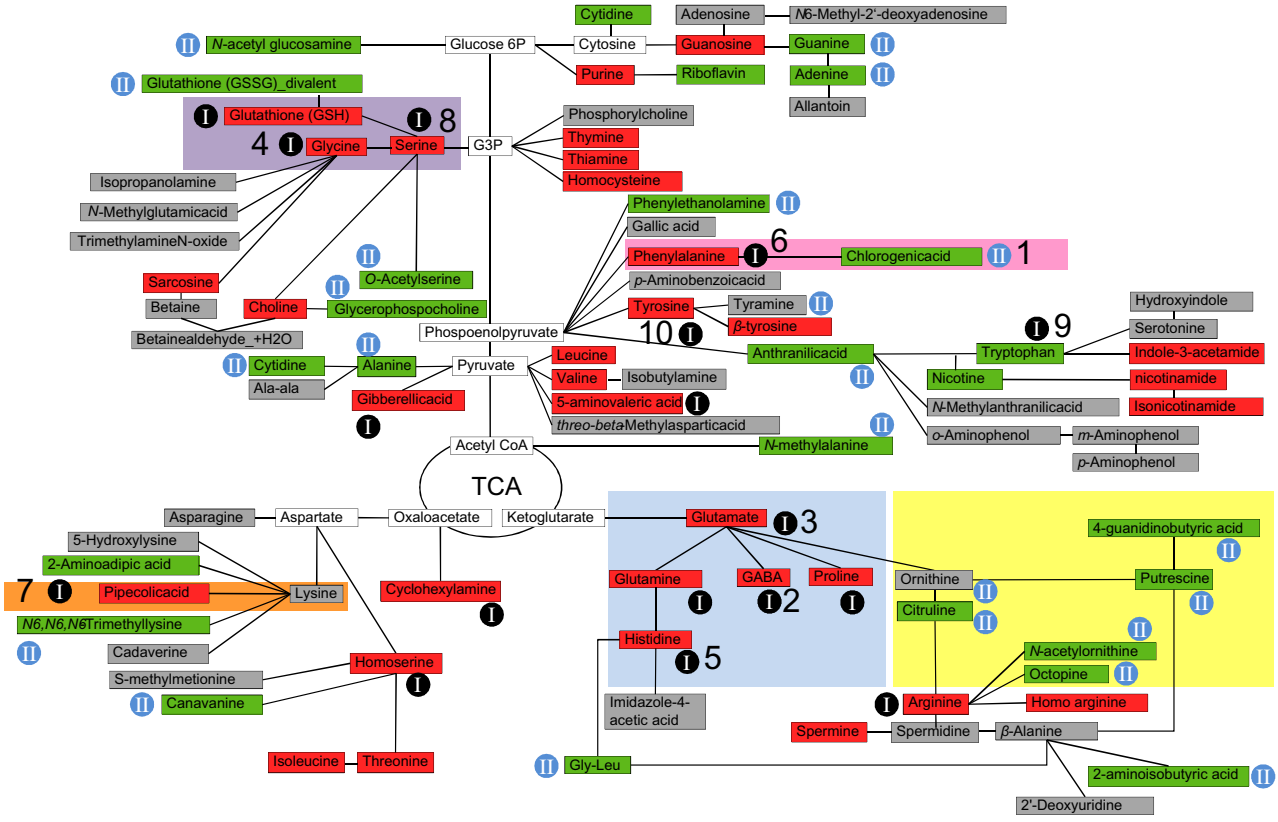

Figure 5. Changes in metabolite levels derived from TCA and glycolysis pathway in 29S inoculated by *R. solani* at average of 1dpi, 2dpi, and 4dpi, based on hierarchical cluster data (Figure 2). Other explanations in the figure were same to Figure 4.

branch on metabolic flow during *R. solani* inoculation was not revealed as an MPP analysis result (Figures 4 and 5). It was appeared that metabolic response to *R. solani* infection in rice plant basically similar in soybean case infected by *R. solani* (Aliferis et al., 2014). The metabolite changes occurred in the entire system of the plants after pathogen infection as plant defence mechanism.

According to Fiehn et al. (2012), the alteration of metabolites is associated with cellular regulatory processes in the plant. In addition Sumner et al. (2003) explained that the metabolite analysis in metabolomics used to infer the gene function. Therefore, it expected that many cellular processes in the rice plant such as genes expression change after *R. solani* infection. Based on the metabolite distribution pattern in the Figures 4 and 5, probably, the cellular processes were not controlled by one gene or a few genes on specific biosynthesis pathway but many genes involved and distributed in random biosynthesis pathway.

Soluble protein and amino acid in rice lines infected by R. solani

Soluble protein and total free amino acid were used to study the response of rice plants against pathogen infection. The soluble protein in mock inoculation of both rice lines at 1 dpi and 2 dpi was significantly higher than that in the inoculated plants (Figure 6). Further, the soluble protein of inoculated 29S was higher than 32R at 4 dpi. Based on the time course changes of soluble protein, inoculated 32R tend to decrease after inoculation. On the other side, the opposite trend was occurred in inoculated 29S.

Total free amino acid of each rice line showed a similar trend in each time course (Figure 6). In other words, the total free amino acid of each line was not affected by the *R. solani* inoculation. However, as indicated in volcano plot analysis (Figure 3), eight amino acids were different among treatment and rice lines. The time courses of 8 amino acids were shown at Figure 7. Specific amino acids; GABA, glutamate, glycine, histidine, phenylalanine, serine, tryptophan, and tyrosine were higher in the 29S compared to those in 32R. It suggests that specific amino acid may associate to the susceptibility of rice plants against pathogen infection. The high concentration of some amino acid in susceptible host plants provides suitable conditions for growth and development of pathogens through nutrient supplies (Seifi et al., 2013). In addition, certain amino acid influences the pathogen development in the mechanism of pathogen infection by act as chemical exudates to attract the germination of *R. solani* (Keijer, 1996).

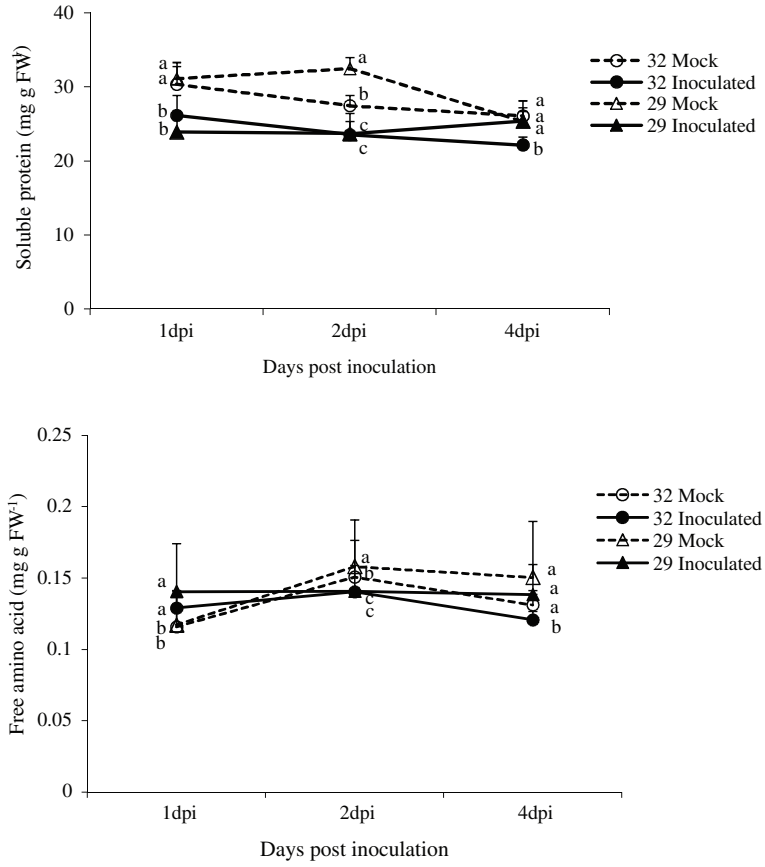

Figure 6. Time course changes of soluble protein and free amino acid (mg gFW^{-1}) on rice leaves at 1dpi, 2dpi, 4dpi in *R. solani* inoculated 32R and 29S (●, ▲), and mock inoculated (○, △). For each time course, values followed by the same letter are not statistically different ($p < 0.05$) by one-way ANOVA test followed by tukey's test hsd (honestly significant different) at each dpi.

The levels of metabolites in GABA, glutamate, and histidine in the 29S were higher than those in 32R during 4 days after *R. solani* inoculation. According to Forde and Lea (2007), GABA synthesis is derived from glutamate. Further, irreversible action of glutamate decarboxylase activity alters glutamate to GABA. Meanwhile, histidine associated with glutamate as *α*-ketoglutarate generator to convert histidine into glutamate (Lemire et al., 2010). Hecker et al. (1975) explained that glutamate deficiency occurs in resistant variety.

Accumulation of GABA in 29S at 2 dpi was occurred after *R. solani* inoculation, those metabolites in mock inoculated 29S and 32R showed a similar pattern with a downward trend after treatments. The accumulation of GABA in plant infected by fungal pathogen contributes to the inactivation of GABA shunt, which induces reactive oxygen species (ROS) activity (Takahashi et al., 2008). According to Bolwell and Daudi (2009), ROS involved in basal resistance, hypersensitive response, and systemic acquired resistance. Nevertheless, necrosis due to ROS activity during pathogen infection, enhance the host susceptibility to necrotrophic (Barna et al., 2012).

The content of phenylalanine, tryptophan, and tyrosine in 29S was higher than those of 32R at 1, 2, and 4 dpi. Those metabolites are important as secondary metabolite precursor derived from chorismate which synthesized through shikimate pathway (Tzin & Galili, 2010). Further, inoculation of *R. solani* increased those metabolites in 29S at 1, 2, and 4 dpi, except tryptophan at 2 dpi. Phenylalanine is suggested to associate with tyrosine. Both metabolites are important in plant defence against biotic stress in phenol synthesis and lignin accumulation which is catalysed by tyrosine ammonia lyase and PAL (Green et al., 1975). Concurrently with phenylalanine and tyrosine, tryptophan is derived from chorismate originates from the shikimate pathway (Wakasa & Ishihara, 2009). Tryptophan is suggested to be involved in plant defence as explained by Sanchez-Vallet et al. (2010) that the genes encoding the enzyme of tryptophan-derived metabolites biosynthesis observed after plant infected by necrotrophic fungi.

The content of glycine and serine in 29S-mock inoculated was higher than those of 32R. The inoculation of *R. solani* increased the level of glycine and serine in 29S at 1 dpi and 4 dpi. Glycine and serine have been suggested

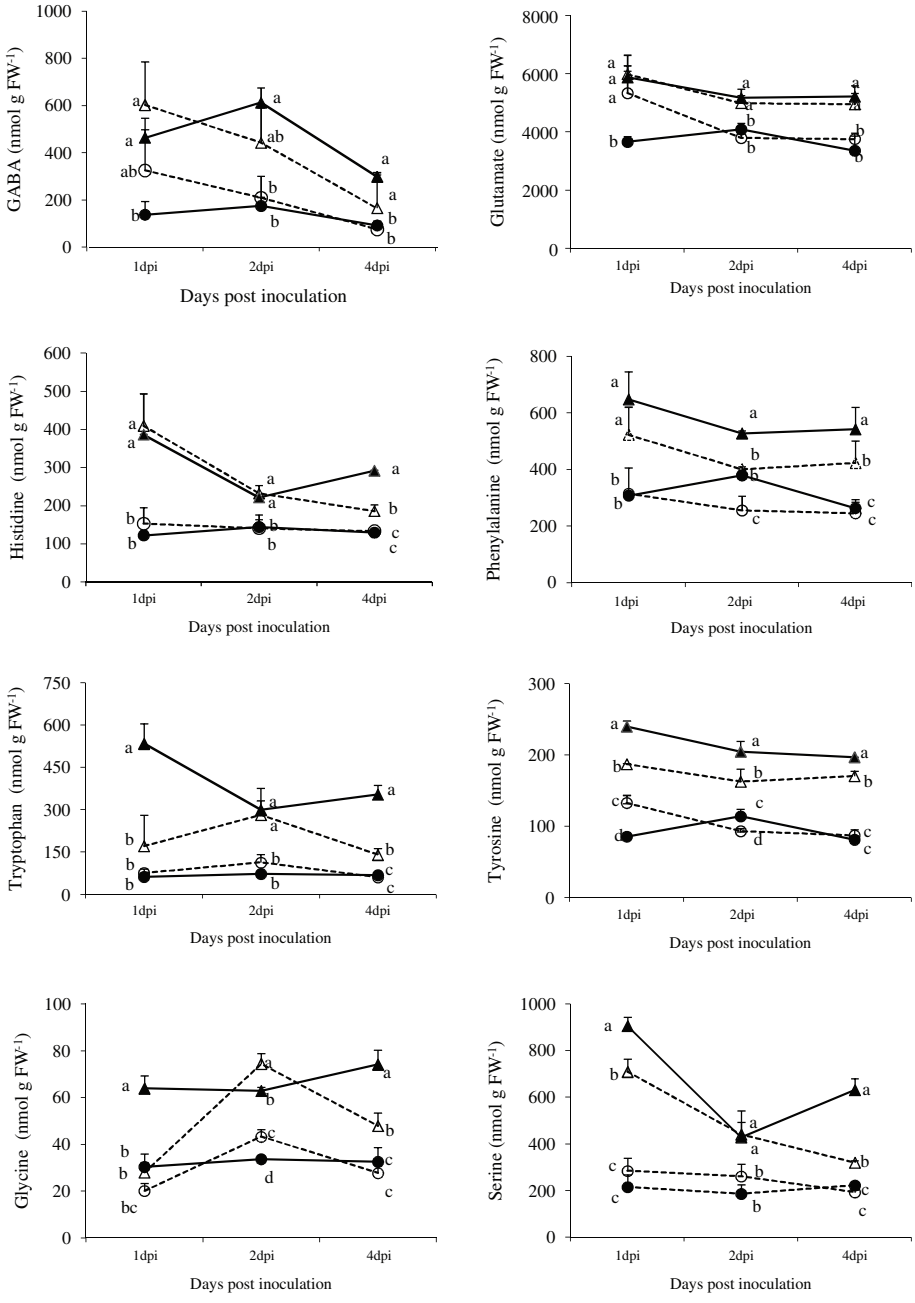

Figure 7. Time course changes of 8 amino acid (nmol g FW^{-1}) on rice leaves at 1dpi, 2dpi, 4dpi in *R. solani* inoculated 32R and 29S (●, ▲), and mock inoculated (○, △). Other explanations about the symbols in the figure were same to Figure 6.

to play a role as plant response to pathogen infection (Tavernier et al., 2007). The changes of glycine and serine in the plant caused by pathogen infection related to the photorespiration system in the leaf. Photorespiration plays a role in plant pathogen interaction, especially in the ROS accumulation (Kangasjärvi et al., 2012).

Phenol involvement in rice lines and R. solani interaction

Two external standards, gallic acid and chlorogenic acid, were assessed by CE/TOF-MS to examine the involvement of phenol in the response of rice lines to infection by the rice sheath blight pathogen. The generated data of gallic acid were unstable. Therefore, the gallic acid is not included.

The analysis results using MPP software showed that chlorogenic acid has a high magnitude fold changes and statistical significant difference among identified metabolite between 32R and 29S rice lines after inoculated by *R. solani* (Figure 3 and Table 1). Chlorogenic acid increased in 32R after inoculation by *R. solani* (Figure 2). Based on the time course, the chlorogenic acid level of 32R was higher than 29S (Figure 8). This suggested that chlorogenic acid

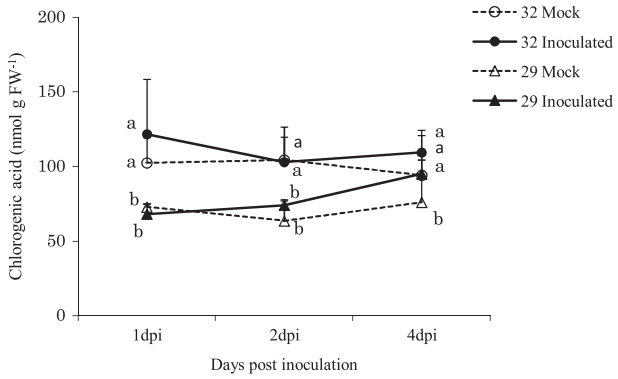

Figure 8. Time course changes of chlorogenic acid (nmol g FW^{-1}) on rice leaves at 1dpi, 2dpi, 4dpi in *R. solani* inoculated 32R and 29S (●, ▲), and mock inoculated (○, △). Other explanations about the symbols in the figure were same to Figure 6.

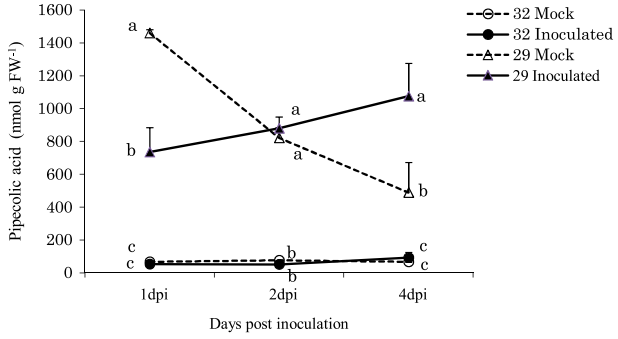

Figure 9. Time course changes of pipecolic acid (nmol g FW^{-1}) on rice leaves at 1dpi, 2dpi, 4dpi in *R. solani* inoculated 32R and 29S (●, ▲), and mock inoculated (○, △). Other explanations about the symbols in the figure were same to Figure 6.

probably contributed as properties owned by 32R as defence mechanism to *R. solani* infection. In the previous study, the level of chlorogenic acid was high in the resistant line to susceptible line against *Alternaria alternata* infection in tomato (Wojciechowska et al., 2014).

As shown in Figure 8, the chlorogenic acid level between mock and inoculated plants in each lines was not significantly different, except in 29S at 4 dpi. In addition, the significant difference of chlorogenic acid in inoculated 32R and 29S was detected at 1 dpi. This result suggested two possibilities that chlorogenic acid of 32 R was originally higher than that of 29S, and physical injury caused by treatments might appear in both rice lines within 1 day after inoculation.

Pipecolic acid in SAR of rice lines to R. solani infection

Pipecolic acid was detected as the metabolite with the highest magnitude of fold change and *p*-value significance level (Figure 3 and Table 1). Pipecolic acid was represented in cluster I of hierarchical cluster (Figure 2). Analysis based on the time course of change by MassHunter software showed the pipecolic acid in 29S was more abundant than that in 32R at 1, 2, and 4 dpi (Figure 9). In the mock inoculated 29S, pipecolic acid levels decreased during the time course of this experiment. In opposite, the increase in pipecolic acid level was observed in the inoculated 29S during the time course. Besides, the levels of pipecolic acid in 32R, both in mock and *R. solani* inoculated were stably low.

Higher level of pipecolic acid in susceptible line observed in this study can be inconsistent with a study reported by Vogel-Adghough et al. (2013) that pipecolic acid involved in resistance mechanism in plants after infected by biotrophic pathogen. Probably, the differences between biotrophic and necrotrophic pathogens affect on the pattern of pipecolic acid accumulation and its role in resistance mechanisms. Pipecolic acid is an essential non-protein amino acid regulating the SAR (Vogel-Adghough et al., 2013) and causing necrotic symptoms as a part of a hypersensitive response (HR) (Conrath, 2006). According to Govrin and Levine (2000), necrotrophic pathogens can promote the HR with different characteristics compared with the response to biotrophic pathogens. As known, *R. solani* is a necrotrophic pathogen which causes necrotic lesions (Park et al., 2008). Therefore, it suggests that HR caused by *R. solani* infection has opposite effects on the plant defence mechanism.

Based on the research results using MassHunter Qualitative software, both rice lines have been influenced by *R. solani* infection at 1 dpi. This is consistent with previous studies conducted in our laboratory (Mutuku & Nose, 2010, 2012), the differences in enzyme activity and metabolite levels of 32R and 29S occurred in 1 dpi. Based on this observation, the future study within one day after inoculation was needed to be done.

Conclusions

CE/TOF-MS is an effective instrument for profiling metabolite in rice plant infected by sheath blight disease because the metabolic pathway responded to the infection were not distributed in a particular pathway in metabolic map, but randomly spread. Plant metabolites, especially amino acids and phenols that involved in plant defence to *R. solani* infection can be detected and characterized by CE/TOF-MS. Based on the study, total amino acid content was found to be similar in the two rice lines. However, some amino acids; glutamate, GABA, glycine, histidine, phenylalanine, serine, tryptophan, tyrosine, and pipecolic acid were abundant in 29S and influenced by the presence of *R. solani*. The enhancement of the specific amino acids in 29S may increase the plant susceptibility as host response

to necrotrophic pathogens. On the other side, chlorogenic acid was primarily higher in 32R. These results suggest that the accumulation of chlorogenic acid could be related to the resistance to pathogen and its level was always maintained high in 32R to prepare for defence against a pathogen infection in advance. Significant differences on amino acid and phenol were detected at first day after *R. solani* inoculation. Therefore, further research is needed to assess the determination of the exact level of each metabolite and time course within one day after inoculation.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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