Metabolomic study on the resistance mechanism of rice sheath blight disease

（メタボローム解析を用いたイネ紋枯病抵抗性メカニズムに関する研究）

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Abbreviations

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
BSA	Bovine serum albumin
CE/TOF-MS	Capillary electrophoresis/time of flight-mass spectrometry
Dpi	Days post inoculation
FC	Fold change
GABA	γ-aminobutyric acid
HR	Hypersensitive response
IMP	Inosine monophosphate
MH	MassHunter
MPP	Mass profiler professional
mRNA	Messenger ribonucleic acid
PSA	Potato sucrose agar
Qtl	Quantitative trait loci
R. solani	*Rhizoctonia solani*
ROS	Reactive oxygen species
TCA	Tricarboxylic acid
UMP	Uridine monophosphate
32R	$2F_{18}$-7-32 (Resistance line)
29S	$2F_{21}$-21-29 (Susceptible line)

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Chapter 1

General introduction

Rice is an economically important food crop as major human diet in several countries in the world; especially Asia (Papademetriou, 2000; Muthayya et al., 2014). Aside from being the largest consumer, Asia also accounts for 90% of world rice production (Tran, 1997). However, abiotic and biotic factor may influence the rice cultivation in decreasing of production. According to Drame et al. (2013), abiotic factors of drought, nutrient limitation, water excess, temperature, and salinity are stress conditions in rice production. Biotic factor, such as weeds, insect and pathogen can causes plant disease as limiting factor in growth production (Ekeleme et al., 2007; Barnwal et al., 2013; Savary et al., 2000).

Rice sheath blight disease is the main diseases in rice cultivation which reported in 1910 (Lee and Rush, 1983). Furthermore, according to Lee and Rush (1983), the yield lost reach up to 50% with symptoms spread to the sheath and leaves of rice plants. The disease is caused by *Rhizoctonia solani* Kuhn known as fungal pathogen, survive as sclerotium or mycelium in plant tissues and spread on the water surface, germinate, and infect the rice plants (Marchetti, 1983). Rice sheath blight disease

resulted in damage of plant tissue with symptoms in the sheath which can spread cause wilting and then death of the leaves (Taheri and Tarighi, 2011). Danson (1999) explained that the symptoms of the plant was shown at 1dpi and further spread to the leaves part of the plant. According to Lee and Rush (1983), rice sheath blight symptoms can be found in young plants with suitable conditions. However, initial symptoms usually appear on lower leaf sheaths at the late tillering or early internode elongation stage. The lesion is showed as 0.5-3 cm circular, oblong, or ellipsoid with green-gray color. The lesion is developed approximately 1 cm in width and 2-3 cm in length. Subsequently, the center will be bleached with an irregular purple-brown border.

Various techniques and strategies, such as chemical, biological, and cultural control have been used as rice sheath blight disease management (Yellareddygari et al., 2014). Biological controls by using *Pseudomonas fluorescens*, *Trichoderma* strains and *Bacillus* are effective to suppress the development of *R. solani* symptoms *in vitro* and greenhouses scale (Nagarajkumar et al., 2004; Naeimi et al., 2010; Ali and Nadarajah, 2013). Chemical control by using Azoxystrobin was effective in suppressing the sheath blight disease development by more than 64% (Sundravadana and Alice, 2007). However, the chemical control is considered toxic and not friendly to environment. Cultural control can be implemented by using varieties with diverse plant resistance to

sheath blight disease. According to Hossain et al. (2014), several rice varieties, such as Teqing, Jasmine 85, Tetep, Pecos, Azucena and Taducan are referred as moderate resistant to the pathogen. Meanwhile, MR 219 is susceptible.

The mechanism of plant resistance against plant pathogens can be divided into vertical and horizontal resistance. Vertical resistance is known as major gene which depends on one or a few genes (Van der Plank, 1966). This unstable resistance is proposed as limiting factor in the plant disease management (Palloix et al., 2009; Sakr, 2010). Horizontal resistance is known as a minor gene resistance. The horizontal resistance is depending on poly or many genes and tends to be durable (Johnson, 1981). According to Zuo et al. (2014), the resistance to rice sheath blight disease is controlled by polygene or quantitave trait loci (QTL's). Number of QTL regions relating to rice sheath blight disease resistance have been discovered and associated with a broad spectrum of quantitave disease resistance (Wisser et al., 2004). Research in our laboratory by Gaihre et al (2015) showed that several QTLs in 32R are resistance to the sheath blight disease.

Previous research in our laboratory, two rice lines, 32R and 29S were bred from Tetep x CN4-4-2 crossing by Wasano et al (1985). Tetep is a cultivar originated from Vietnam with some resistance to sheath blight disease. Meanwhile, CN4-4-2 was a

hybrid from Chugoku 45 and Nipponbare, a japonica variety with susceptibility to sheath blight disease. The characteristic of 32R is determined as a resistant line, while 29S is identified as a susceptible line to *R. solani* infection. According to Gaihre and Nose (2013), 32R is known as the resistant rice line with yields (7.9 MT/ha) lower than the 29S (8.8 MT/ha). Furthermore, Kiet and Nose (2015) suggested that 29S is well adapted in the cold temperature regime. Both rice lines showed different response to the *R. solani* infection. Danson (1999) showed the changes in carbohydrate metabolism and related enzymes in the rice lines due to *R. solani* infection. In addition, the change of metabolites and lignin accumulation were observed by Danson (1999); Mutuku and Nose (2010; 2012). These studies revealed the evidence of the primary and secondary metabolite involvement in the resistance mechanism of plant due to *R. solani* infection.

Primary metabolites involving to resistance due to pathogen infection will affect to the metabolic processes in plants (Rojas et al., 2014). Furthermore, it was mentioned that the role of primary metabolites as plant defence against pathogen infection associated with carbohydrate, photorespiration, and amino acid metabolism. The correlation between plant defence mechanism and carbohydrate was observed in barley infected by leaf blight disease (Singh et al., 2009) and soybean seedling infected by *R. solani* (Aliferis et al., 2014). The effect of pathogen infection in carbohydrate

metabolism appeared in the metabolites and accumulation of sugar hexose (Aliferis et al., 2014; Tauzin and Giardina, 2014). Photorespiration as the plant defence role was observed in banana infected by *Fusarium oxysporum* f. sp. *cubense* (Dong et al., 2016). Amino acids in primary metabolisms, such as histidine, glycine, serine, tyrosine, and phenylalanine were altered as a result of banana plant response to *Fusarium* infection (Navajothy et al., 2011). In addition, amino acid alterations were appeared in sunflower infected by *Botrytis cinerea* (Dulermo et al., 2009). Dulermo et al. (2009) reported that necrotrophic pathogens acts as a nitrogen sink for amino acid changes during the infection process.

According to Rojas et al. (2014), secondary metabolites involved in plant defence mechanisms due to pathogen infection (Rojas et al., 2014). Generally, secondary metabolites involving in the defence mechanism are phenolic compounds. It may lead to the lignin formation as a final product. According to Akhtar et al. (2011), phenolic compound is widely mentioned as metabolic role in plant resistance. As explained by Cheynier (2012), phenol has structures ranging from simple molecules, polyphenols and polymers. It may promote the lignin accumulation as plant defence to pathogen infection. Lignin is the main component of cell wall that can be used as first defence mechanism in the penetration of fungal pathogen (Bhuiyan et al., 2009).

Furthermore, several phenolic compounds have antifungal activities against fungal pathogen of plant (Ansari et al., 2013).

In this study, we investigated responses of resistant and susceptible rice lines due to *R. solani* infection with metabolomic approach by using CE/TOF-MS. Metabolomic is a part of omics for analyzing the plant tissue with simple, accurate, and time saving. As general, omics is the technology with primarily objective to detect the genes (genomics), mRNA (transcriptomics), proteins (proteomics) and metabolites (metabolomic) on a biological sample (Horgan and Kenny, 2011). Omics studies will provide a holistic and comprehensive observation on plants (Tohge et al., 2005; Hall et al, 2002). According to Dettmer et al. (2007), metabolomic have a purpose to analysis quantitatively and comprehensively various metabolites of the sample. It is used to determine the low molecular weight metabolites of the tissue samples in comprehensively (Sugimoto et al., 2014). By using metabolomic analysis, primary metabolites which essensial for plant cell growth and development is observed. In addition, a secondary metabolite as a plant signaling, hormones, and the defense mechanism can be acknowledged.

The metabolomic study can be implemented by 4 kinds of approaches; the target analysis, metabolomics, metabolic fingerprinting, and metabolite profiling

(Roessner and Bowne, 2009). Furthermore, the targeted metabolomic analysis can be conducted when the metabolite is already determined. The metabolomic approach is applied by using the complementary methods of analysis to determine and quantify the metabolites, both target and non-target metabolites (Roessner and Bowne, 2009). Metabolic fingerprinting is an analytical technique for sample classification based on the bases of the sample origin or the biological relevance (Liang et al., 2015). The last approach, metabolite profiling is used to obtain the complete information, both identified and an unknown metabolites of the sample (Roessner and Bowne, 2009; Wolfender et al., 2015).

Metabolite profiling can be performed by using capillary electrophoresis (CE) equipped with a time of flight (TOF) and mass spectrometry (MS). This instrument is able to generate metabolomic data. Based on the type of analysis, metabolite profiling research using capillary electrophoresis conducted with two types of ion mode, positive and negative ion mode. Both types of ion modes produce the number of primary and secondary metabolites which play a role in the metabolism of rice plants. According to Ahmad (2013), positive ion mode is suitable for cationic metabolites detection, such as amino acids, phenolic, carboxylic, thiol, and phosphate/sulfate group. In other side, Soga et al. (2003) and Ahmad (2013) explained that negative ion mode

analysis is appropriate for anionic metabolites, such as sugar phosphate, organic acid, Coenzyme A compounds, and nucleotides. In Chapter 2 and 3, metabolomic study has successfully revealed the cationic and anionic metabolite by using positive and negative ion mode in CE/TOF-MS.

CE-TOF/MS consists of Capillary Electrophoresis coupled with Mass Spectrophotometer and Time of Flight which is a valuable instrument for a number of studies on the metabolite research. Sato et al. (2004) has been using CE/TOF-MS for metabolomic research on rice leaves. The combination provides efficient and accurate results in separation of low molecular metabolite sample (Staub et al, 2009). Since 2008, CE-TOF/MS appearanced a rapid development in application and applied in the diagnosis of clinical research, also molecular field (Kolch et al., 2005; Sugimoto, 2010; Ramautar et al., 2011). Capillary electrophoresis (CE) is ions separation technique of both small and large molecule based on electrophoretic mobility by using an applied voltage. As a simple explanation, the instrument is consisted of a high-voltage power supply, a sample introduction system, a capillary tube, a detector and an output device. The mode of ion separation divided into 2 types; positive and negative ion mode. Positive ion mode generally determines amino acid (Sato et al., 2004). CE/TOF-MS by using negative ion mode have been used by Soga et al (2009) to obtain primary

metabolites.

Metabolomic study will provide the information of resistance mechanisms on rice lines due to *R. solani*. It is very useful as additional source of information in the engineering of rice resistant variety to sheath blight disease. Based on those objectives, this study is consisted of 5 Chapters; general introduction, metabolite profiling of sheath blight disease resistance in rice: in the case of positive ion mode analysis by CE/TOF-MS, metabolomic study of two rice lines infected by *R. solani* in negative ion mode by CE/TOF-MS, canavanine involvement in the interaction of rice lines and *R. solani*, and general discussion.

Chapter 2

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

1. Introduction

Rice sheath blight disease, caused by *Rhizoctonia solani* is a major disease affecting rice cultivation and distributed all over the world (Ou, 1985). *R. solani* is a necrotroph pathogen of necrotic lesions on rice plant that affects plant growth (Park et al., 2008; Simonetta et al., 2007). The symptom in infected plant appears on the sheath and spreads to the leaves. The disease decreases the rice yield by 50% in the field (Lee and Rush, 1983). Cultural practices and, biological and chemical controls have been developed to ameliorate the problem (Rodrigues et al., 2003; Slaton et al., 2003; Nagarajkumar et al., 2004). 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).

The breeding of sheath blight disease resistant varieties has been hindered due to the lack of a resistant major gene (Zeng et al., 2011). Major genes provide complete resistance to plants, but are less stable and easy to break down (Agrios, 1988). According to Zou et al. (2000), the resistance of sheath blight disease in rice is

controlled by a polygene. This type of resistance is more stable and durable in the environment (Wasano and Dhanapala, 1982; Parlevliet, 2002). 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. 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 x 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 and 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 and 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 and Nose, 2012). Phenylpropanoid and shikimate pathways are involved in plant defense mechanism (Dixon et al., 2002; Tzin and 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 and 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 defense mechanisms used in the response of 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 and Nose, 2012). It has also been reported that the level of amino acids change during pathogen infection (Aliferis and 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.

2. 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 CN_4-4-2 (*Japonica*). Tetep is a high resistant variety of rice originating in Vietnam, whereas CN_4-4-2 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 $\mu mol\ m^{-2}s^{-1}$ of photosynthetic photon flux density at the plant level) until 7^{th} leaf growth

stage. the plants were inoculated with the pathogen mycelium and transferred to a 28°C incubator (NK System Biotron, Japan). The nutrient solution, mixed with Otsuka House No.1 and No.2 (Otsuka Chemical Co. Ltd., Japan) were applied as inorganic fertilizers. Plant watering was done as needed. The 7^{th} growth leaf stage of rice plants was inoculated by *R. solani* mycelium and maintained in 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 (BSA) as the standard for the assay. Leaf powder (200 mg) was added to 50mM 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 nynhidrin reagent consist of 10 mg mL^{-1} nynhidrin in 0.5M 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 for cation is produced by Human Metabolome Technologies Inc., Japan (HMT). It contains methionine sulfone and champor-10-sulfonic acid for the MS quantification (Takahashi and Washio, 2011). The rice leaf powder (30 mg) was plunged into mixture solution (500 μL) containing 10 mL methanol and 5 μL internal standard solution 1 (HMT). 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 cutoff 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 (HMT), and then vortexed (30 s) and flash centrifuged. The internal standard 3 contains trimesic acid and 3-hydroxynaphtalene-2, 7-disulfonic acid was used for calibration of CE migration time (Takahashi and 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 metabolite concentration was determined based on relative area as a result of Masshunter Qualitative software which equipped with annotation by KEGG (*Kyoto Encyclopedia Genes and Genomes*). External standards were used individually in the research. The external standard was prepared by dissolving gallic acid (10 μg μL^{-1}) and chlorogenic acid (1 μg μL^{-1}) (WAKO, Japan) in distilled water. Single-level calibration of the external standard is conducted by injecting the standards to CE/TOF-MS (Frazier et al., 2000). Subsequently, the concentration and peak area of the standards are applied in the calculation of gallic acid and chlorogenic acid concentration on the sample.

Capillary electrophoresis (Agilent 7100 CE, Agilent Technologies, USA) equipped with Agilent G6224A time- of- flight -mass spectrometry (CE/TOF-MS) was used in the experiment in positive ion mode. The Agilent 1100 isocratic high-performance liquid chromatography (HPLC) pump, an Agilent G1603A CE-MS adapter kit and CE-ESI-MS sprayer kit (G1607A-60041) equipped with stainless steel

ESI sprayer needle from Agilent were employed in the research. A fused silica capillary (50 μm i.d. x 80 cm total length) (HMT) was used for sample separation with buffer solution (HMT) 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.

3. 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 (Fig. 2.1).

The results of the CE/TOF-MS experiment was further analysed by Mass Profiler Professional (MPP). MPP analysis software from Agilent is designed to explore the information contained within the mass spectrometry data and perform excellent statistical analysis. Each identified metabolite from 32R and 29S treated with mock and *R. solani* was visualized by hierarchical cluster analysis (Fig. 2.2). 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 6 clusters at level dissimilarity 2.2 by hierarchical cluster based on Euclidean distance (Fig. 2.2). 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 (Fig. 2.3). 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 (Fig. 2.3 and Table 2.2). 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 (Fig. 2.2).

The changes in metabolite level in inoculated 32R and 29S by *R. solani* in the metabolic map were shown in Fig. 2.4 and 2.5. Casting a glance, the distribution of metabolites in the metabolic map was observed as a random spread in the branches of 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 Figs. 2.4 and 2.5. In 32R,

chlorogenic acid showed a positive response to pathogen infection. Meanwhile, opposite appearance was occurred in the 29S.

As a second property, glutamate was shown as down-regulated metabolite in comparison between inoculated 32R and 29S (Fig. 2.3). 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 Fig. 2.4 and 2.5, 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 (Figs. 2.4 and 2.5). 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 labeling is more effective to trace the metabolite element in cells.

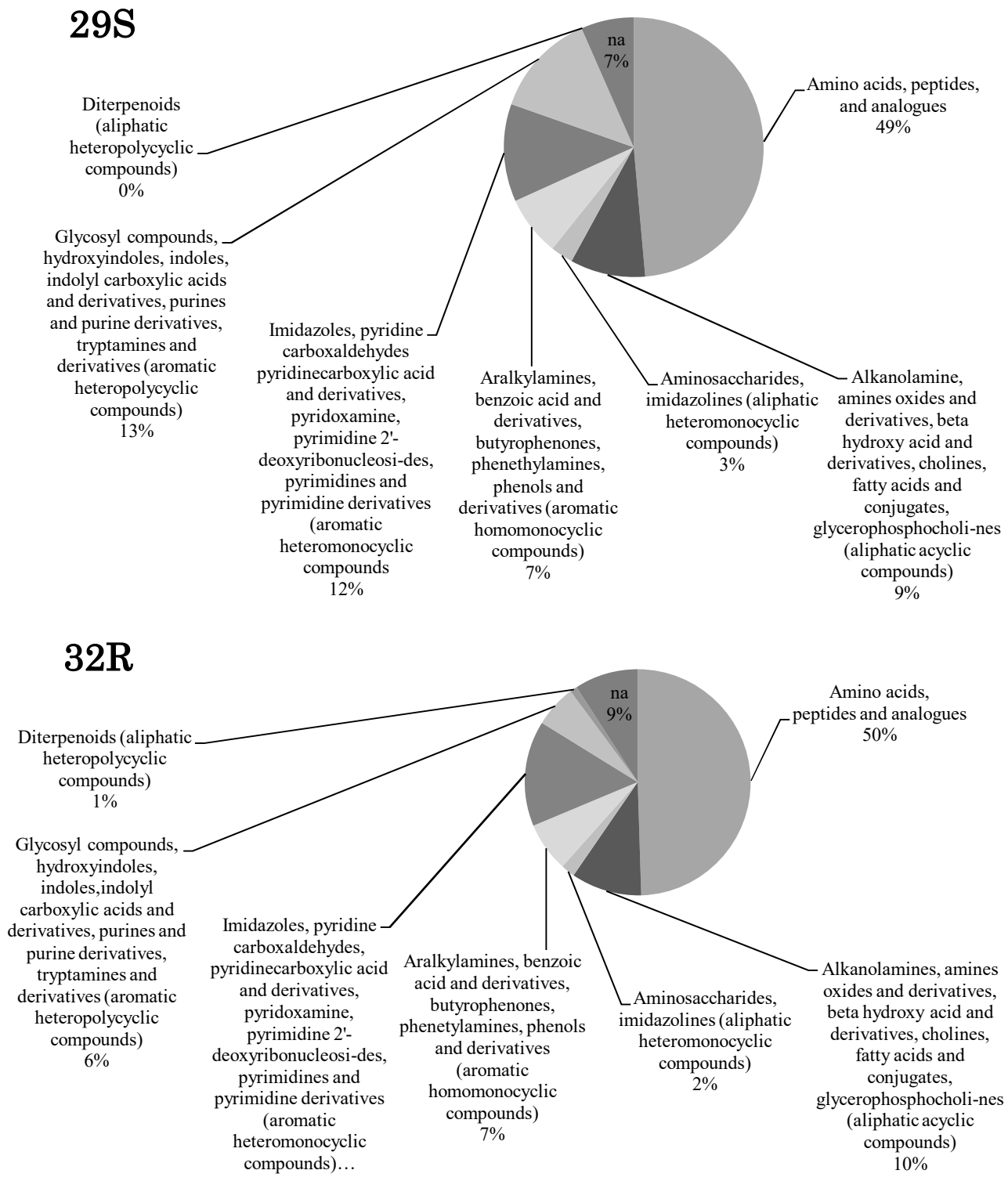

Fig. 2.1. Compound grouping of metabolites in 29S and 32R based on chemical taxonomy.

Table 2.1. Chemical taxonomy of 100 metabolites from hierarchical cluster based on public database

No.	Metabolites	Sub Class	Molecular Framework
1	Pipecolic acid	Amino acids, peptides analogues	Aliphatic heteromonocyclic compounds
2	GABA	Amino acids, peptides analogues	Aliphatic acyclic compounds
3	Glutamate	Amino acids, peptides analogues	Aliphatic acyclic compounds
4	Histidine	Amino acids, peptides analogues	Aliphatic acyclic compounds
5	Glutamine	Amino acids, peptides analogues	Aliphatic acyclic compounds
6	Phenylalanine	Amino acids, peptides analogues	Aromatic homomonocyclic compounds
7	Tyrosine	Amino acids, peptides analogues	Aromatic homomonocyclic compounds
8	Serine	Amino acids, peptides analogues	Aliphatic acyclic compounds
9	Glycine	Amino acids, peptides analogues	Aliphatic acyclic compounds
10	Trypthophan	Amino acids, peptides analogues	Aromatic heteropolycyclic compounds
11	Chloramphenicol	Carboxylic acid derivatives	Aromatic homomonocyclic compounds
12	Arginine	Amino acids, peptides analogues	Aliphatic acyclic compounds
13	Cyclohexylamine	n.a	Aliphatic homomonocyclic compounds
14	Gibberelic acid	Diterpenoid	Aliphatic heteropolycyclic compounds
15	Glutathione (GSH)	Amino acids, peptides analogues	Aliphatic acyclic compounds
16	Homoserine	Amino acids, peptides analogues	Aliphatic acyclic compounds
17	5-Aminovaleric acid	Amino acids, peptides analogues	Aliphatic acyclic compounds
18	Proline	Amino acids, peptides analogues	Aliphatic heteromonocyclic compounds
19	2-Aminoisobutyric acid	Amino acids, peptides analogues	Aliphatic acyclic compounds
20	*N*-Methylalanine	Amino acids, peptides analogues	Aliphatic homomonocyclic compounds
21	Cytidine	Nucleosides, nucleotides and analogue	Aromatic heteromonocyclic compounds
22	Disulfiram	Organic thiocarbonic acid derivative	Aliphatic acyclic compounds
23	Putrescine	Primary amines	Aliphatic acyclic compounds
24.	Nalpha-Benzenol arginine ethylester	n.a	n.a
25.	*O*-Acetylserine	Amino acids, peptides analogues	Aliphatic acyclic compounds
26	Phenylethanolamine	Aralkylamines	Aromatic homomonocyclic compounds
27	Alanine	Amino acids, peptides analogues	Aliphatic acyclic compounds
28	Glycerolphosphate	Glycerolphosphate	Aliphatic acyclic compounds
29	Canavanine	Amino acids, peptides analogues	Aliphatic acyclic compounds
30	*N*-Acetylornithine	Amino acids, peptides analogues	Aliphatic acyclic compounds
31	Glutathione (GSSG)_divalent	Amino acids, peptides analogues	Aliphatic acyclic compounds
32	N6,N6,N6-Trimethyllysine	Amino acids, peptides analogues	Aliphatic acyclic compounds
33	Tyramine	Phenethylamines	Aromatic homomonocyclic compounds
34	Adenine	Purines and purine derivatives	Aromatic heteropolycyclic compounds
35	4-Guanidinobutyric acid	Amino acids, peptides analogues	Aliphatic acyclic compounds
36	Anthranilic acid	Benzoic acid and derivatives	Aromatic homomonocyclic compounds
37	Chlorogenic acid	Cyclic alcohol and derivatives	Aromatic homomonocyclic compounds

No.	Metabolites	Sub Class	Molecular Framework
38	Guanine	Purines and purine derivatives	Aromatic heteropolycyclic compounds
39	Octopine	n.a	n.a
40	Citrulline	Amino acids, peptides analogues	Aliphatic acyclic compounds
41	*N*-Acetylglucosamine	Aminosaccharides	Aliphatic heteromonocyclic compounds
42	Gly-Leu	Amino acids, peptides analogues	Aliphatic acyclic compounds
43	Ornithine	Amino acids, peptides analogues	Aliphatic acyclic compounds
44	1-Aminocyclopentanecarboxylic acid	Amino acids, peptides analogues	Aliphatic homomonocyclic compounds
45	Adenosine	Nucleosides, nucleotides and analogue	Aromatic heteropolycyclic compounds
46	*β*-Alanine	Amino acids, peptides analogues	Aliphatic acyclic compounds
47	Imidazole-4-acetic acid	Imidazoles	Aromatic heteromonocyclic compounds
48	Lysine	Amino acids, peptides analogues	Aliphatic acyclic compounds
49	Indole-3-acetamide	Indoles	Aromatic heteropolycyclic compounds
50	N5-Ethylglutamine	Amino acids, peptides analogues	Aliphatic acyclic compounds
51	Homocysteine	Amino acids, peptides analogues	Aliphatic acyclic compounds
52	beta-Leucine	Amino acids, peptides analogues	Aliphatic acyclic compounds
53	Purine	Purines and purine derivatives	Aromatic heteropolycyclic compounds
54	Glycylglycine	Amino acids, peptides analogues	Aliphatic acyclic compounds
55	Homoarginine	Amino acids, peptides analogues	Aliphatic acyclic compounds
56	Thiamine	Pyrimidines and pyrimidine derivatives	Aromatic heteromonocyclic compounds
57	Choline	Cholines	Aliphatic acyclic compounds
58	Leucine	Amino acids, peptides analogues	Aliphatic acyclic compounds
59	N6-Methyl-2'-deoxyadenosine	n.a	n.a
60	Octylamine	n.a	Aliphatic acyclic compounds
61	Isoleucine	Amino acids, peptides analogues	Aliphatic acyclic compounds
62	Sarcosine	Amino acids, peptides analogues	Aliphatic acyclic compounds
63	Gallic acid	Benzoic acid and derivatives	Aromatic homomonocyclic compounds
64	*S*-Methylmethionine	Amino acids, peptides analogues	Aliphatic acyclic compounds
65	*β*-methylaspartate	Beta hydroxy acid and derivatives	Aliphatic acyclic compounds
66	Isonicotinamide	Pyridinecarboxylic acid and derivatives	Aromatic heteromonocyclic compounds
67	Nicotinamide	Pyridinecarboxylic acid and derivatives	Aromatic heteromonocyclic compounds
68	3-Methyladenine	Purines and purine derivatives	Aromatic heteropolycyclic compounds
69	2-Methylserine	Amino acids, peptides analogues	Aliphatic acyclic compounds
70	Threonine	Amino acids, peptides analogues	Aliphatic acyclic compounds
71	Guanosine	Nucleosides, nucleotides and analogue	Aromatic heteropolycyclic compounds
72	Valine	Amino acids, peptides analogues	Aliphatic acyclic compounds
73	*β*-Tyrosine	Amino acids, peptides analogues	Aliphatic acyclic compounds
74	Thymine	Pyrimidines and pyrimidine derivatives	Aromatic heteromonocyclic compounds

No.	Metabolites	Sub Class	Molecular Framework
75	Isobutylamine	Primary amines	Aliphatic acyclic compounds
76	2-Aminoadipic acid	Amino acids, peptides analogues	Aliphatic acyclic compounds
77	Allantoin	Imidazolines	Aliphatic heteromonocyclic compounds
78	Nicotine	n.a	Aromatic heteromonocyclic compounds
79	Ala-Ala	Amino acids, peptides analogues	Aliphatic acyclic compounds
80	*N*-Methylglutamic acid	Amino acids, peptides, and analogues	Aliphatic acyclic compounds
81	*N*-methylanthranilate	Benzoic acid and derivatives	Aromatic homomonocyclic compounds
82	Isopropanolamine	Alkanolamines	Aliphatic acyclic compounds
83	Trimethylamine N-oxide	Amines oxides and derivatives	Aliphatic acyclic compounds
84	Aspargine	Amino acids, peptides analogues	Aliphatic acyclic compounds
85	*p*-Aminophenol	phenols and derivatives	Aromatic homomonocyclic compounds
86	Betaine aldehyde_+H2O	n.a	Aliphatic acyclic compounds
87	Spermine	Secondary amines	Aliphatic acyclic compounds
88	Hydroxyindole	Hydroxyindoles	Aromatic heteropolycyclic compounds
89	Riboflavin	Alloxazines and isoalloxazines	Aromatic heteropolycyclic compounds
90	Tyrosine methyl ester	Amino acids, peptides analogues	Aromatic homomonocyclic compounds
91	2'-Deoxyuridine	Nucleosides, nucleotides and analogue	Aromatic heteromonocyclic compounds
92	Spermidine	Secondary amines	Aliphatic acyclic compounds
93	Betaine	Amino acids, peptides analogues	Aliphatic acyclic compounds
94	*p*-Aminobenzoic acid	Benzoic acid and derivatives	Aromatic homomonocyclic compounds
95	Serotonin	Tryptamines and derivatives	Aromatic heteropolycyclic compounds
96	5-Hydroxylysine	Amino Acids, Peptides, and Analogues	Aliphatic acyclic compounds
97	Phosphorylcholine	Cholines	Aliphatic acyclic compounds
98	Cadaverine	Primary amines	Aliphatic acyclic compounds
99	*m*-Aminophenol	Phenols and derivatives	Aromatic homomonocyclic compounds
100	*o*-Aminophenol	Phenols and derivatives	Aromatic homomonocyclic compounds

n.a; not available

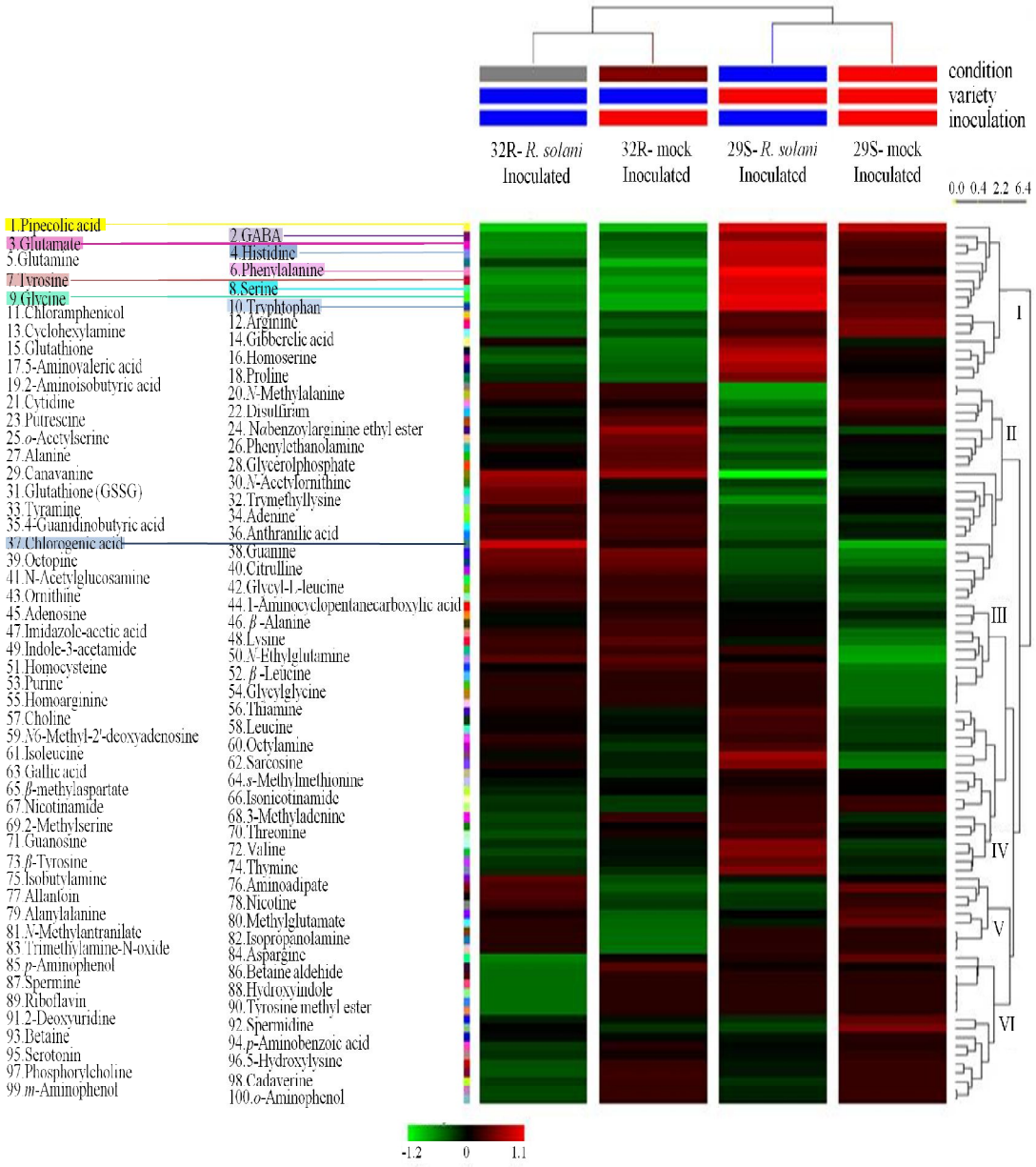

Fig. 2.2. Hierarchical cluster analysis of identified cation metabolite on susceptible (29S) and resistant (32R) rice lines between *R. solani* inoculated and mock control at average of 1dpi, 2dpi, and 4dpi. Chlorogenic acid and gallic acid as phenol standard was added as external standard. Color intensity related to the degree of increase (red) and decrease (green) of the mean metabolite ratio. The Romans numeral referred to the cluster number.

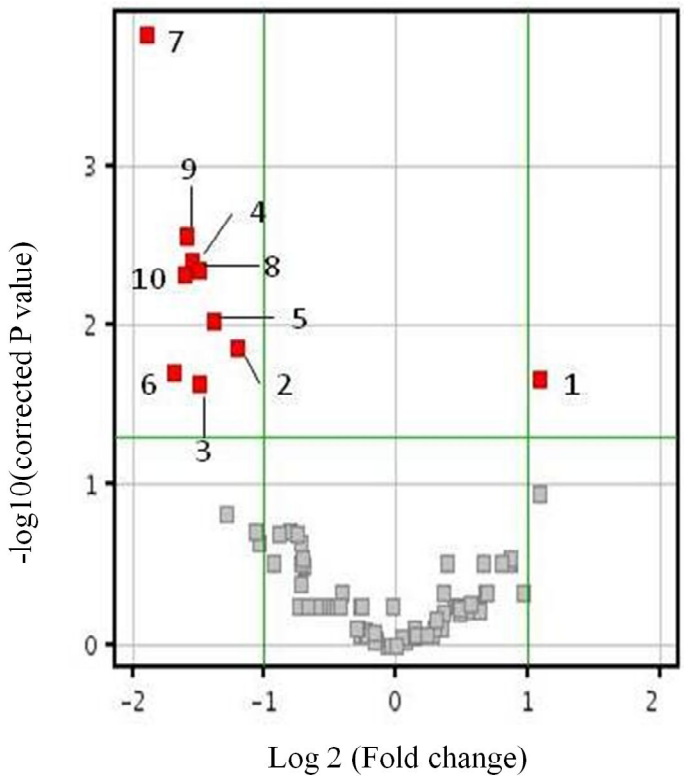

Fig. 2.3. Volcano plot analysis illustrated of identified metabolite between resistant (32R) and susceptible (29S) rice lines after inoculated by *R. solani* at average of 1dpi, 2dpi, and 4dpi. Red square represents the metabolite displayed with larger magnitude fold-changes (x-axis, FC≥2.0) and statistical significance difference (y-axis, P≤0.05). Metabolites numbering in volcano plot are accordance with metabolites number as listed in Table 2.1.

Table 2.2. 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.

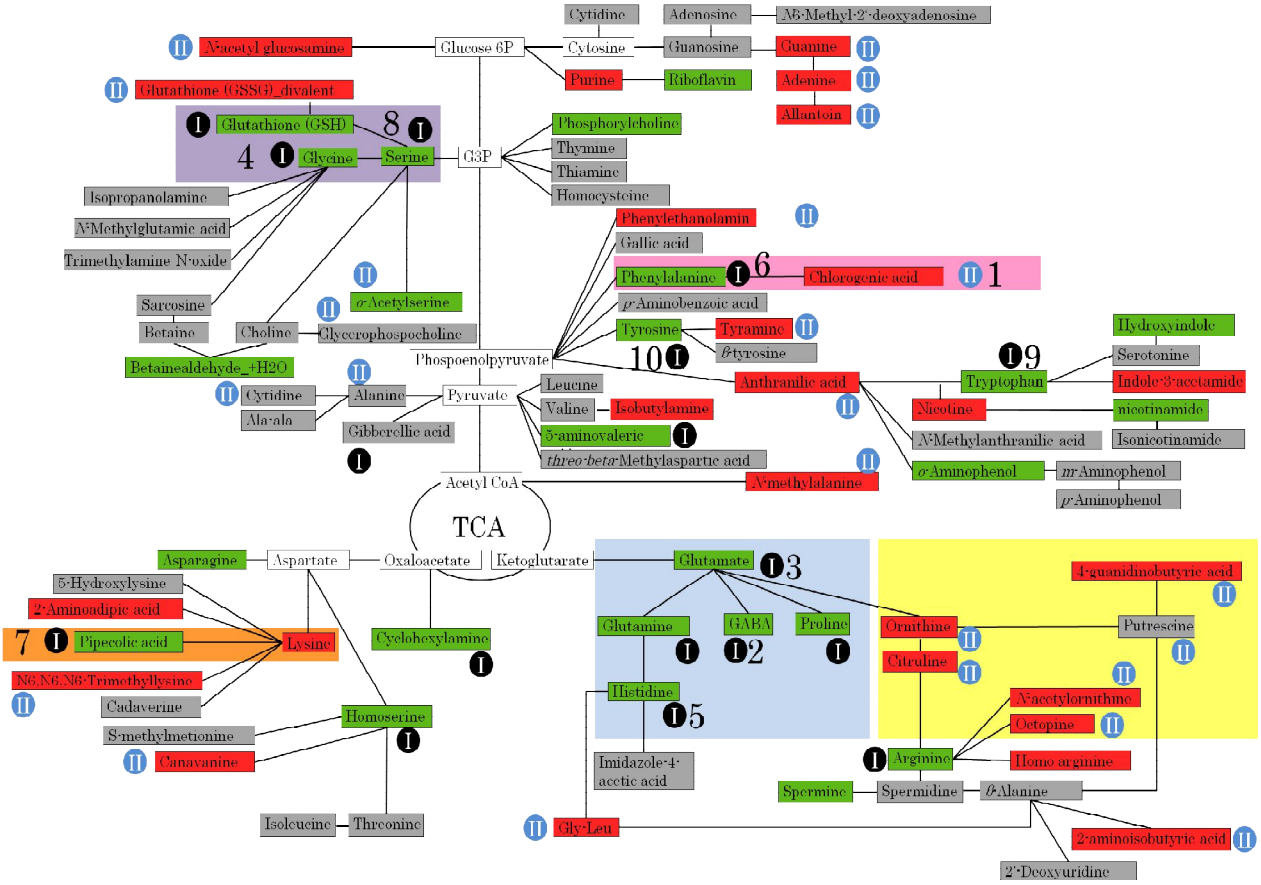

Fig. 2.4. Changes in metabolite levels derived from TCA and glycolysis pathway in 32R inoculated by *R. solani* at average of 1dpi, 2dpi, and 4dpi, based on hierarchical cluster data (Fig. 2.2). Increased and decreased intensity of metabolites were showed in red and green colour. The Romans numeral (I 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.

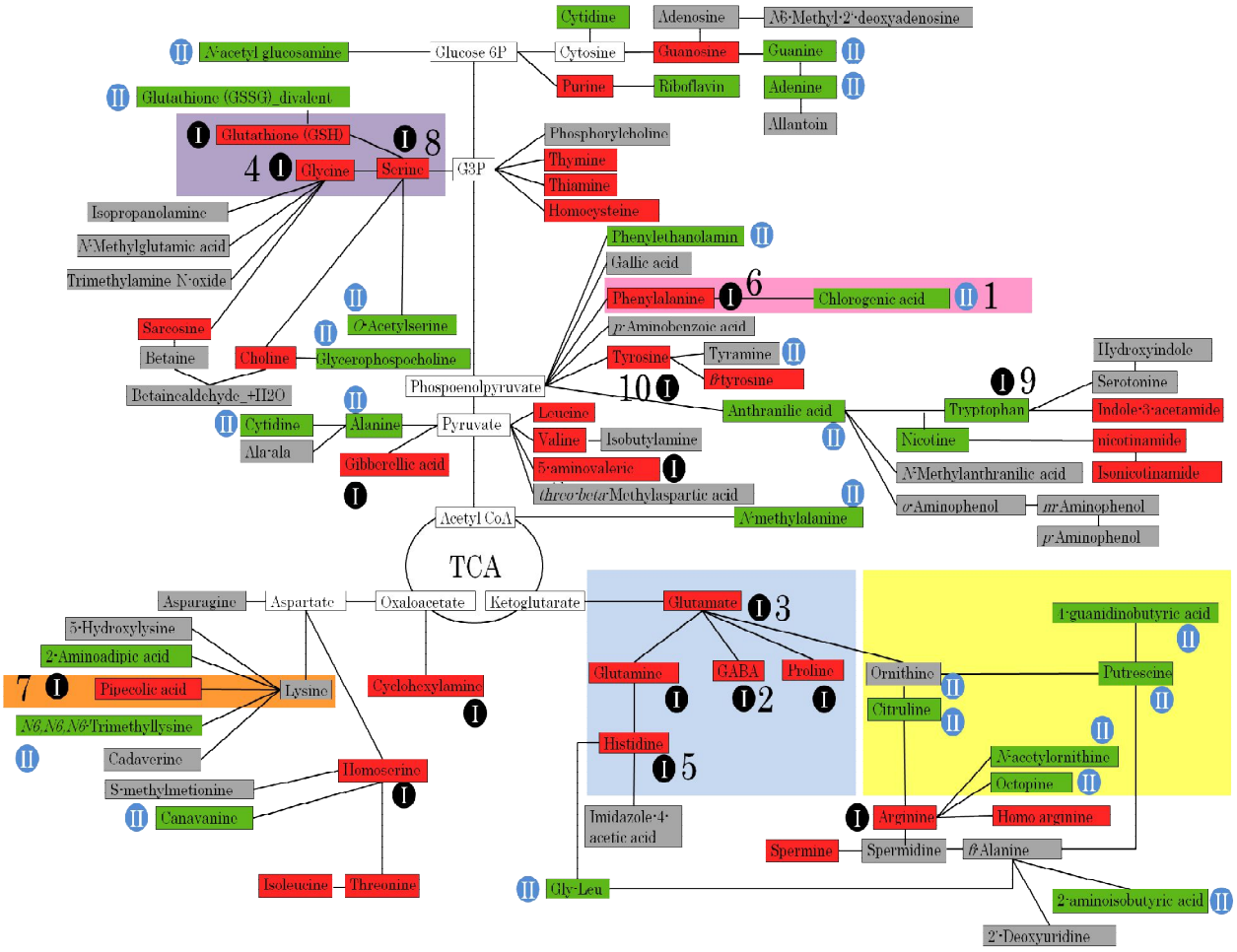

Fig. 2.5. Changes in metabolite levels derived from TCA and glycolysis pathway in 29S inoculated by *R. solani* at average of 1dpi, 2dpi, and 4dpi, based on hierarchical cluster data (Fig. 2.2). Other explanations in the figure were same to Fig. 2.4.

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 (Fig. 2.3). Level of pipecolic acid was detected abundantly in inoculated 29S than inoculated 32R (Figs. 2.4 and 2.5). 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 MassHunter Qualitative data is continued in the next section.

Serine, glycine, glutathione (GSH) was derived from glycerate 3 phosphate (G3P) (Figs. 2.4 and 2.5). 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 (Fig. 2.2).

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 branch on metabolic

flow during *R. solani* inoculation was not revealed as an MPP analysis result (Fig. 2.4 and 2.5). 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 defense mechanism. According to Fiehn (2002), 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 were change after *R. solani* infection. Based on the metabolite distribution pattern in the Fig. 2.4 and 2.5, 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 (Fig. 2.6). 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 (Fig. 2.6). 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 (Fig. 2.3), eight amino acids were different among treatment and rice lines. The time courses of 8 amino acids were shown at Fig. 2.7. 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).

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 converts 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 and 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 defense against biotic stress in phenol synthesis and lignin accumulation which is catalyzed by

tyrosine ammonia lyase and phenylalanine ammonia lyase (Green et al., 1975). Concurrently with phenylalanine and tyrosine, tryptophan is derived from chorismate originates from the shikimate pathway (Wakasa and Ishihara, 2009). Tryptophan is suggested to be involved in plant defense 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 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.

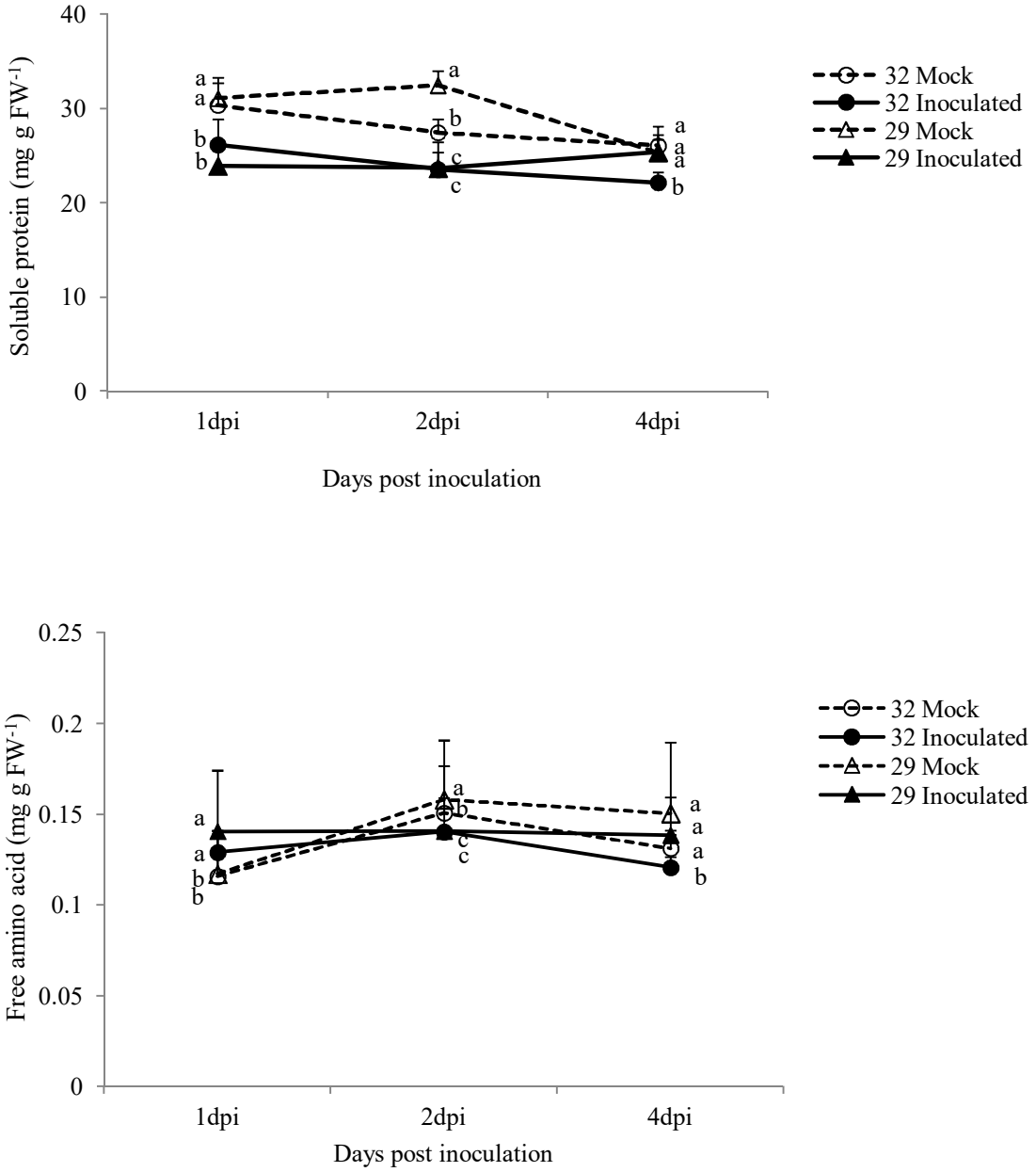

Fig. 2.6. Time course changes of soluble protein and free amino acid (mg gFW^{-1}) on rice leaves at 1dpi, 2dpi, 4dpi in *R solani* inoculated 32R and 29S (●, ▲), and mock inoculated (○, △). For each time course, values followed by the same letter are not statistically different ($p<0.05$) by one-way ANOVA test followed by tukey's test hsd (honestly significant different) at each dpi.

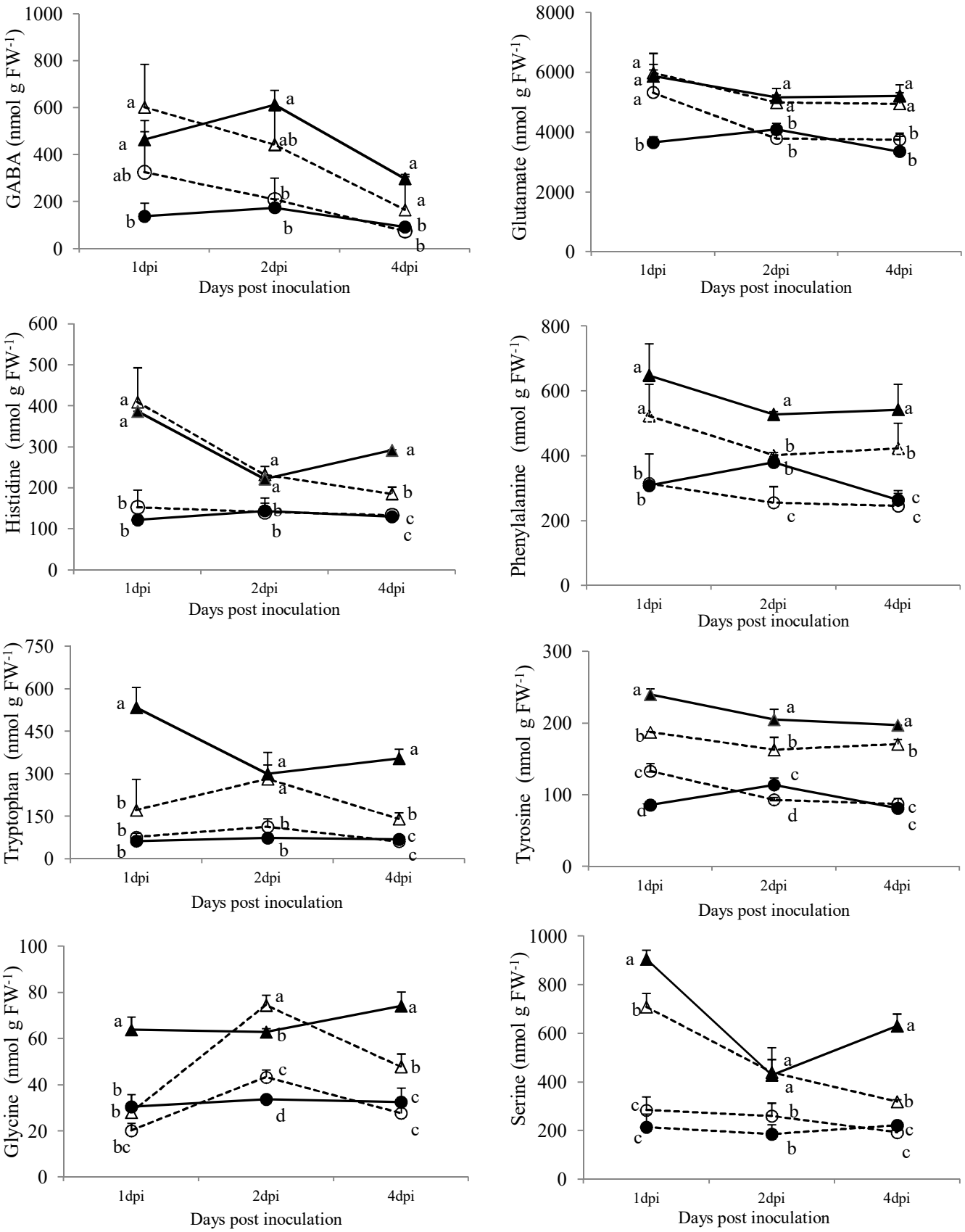

Fig. 2.7. Time course changes of 8 amino acid (nmol g FW^{-1}) on rice leaves at 1dpi, 2dpi, 4dpi in *R solani* inoculated 32R and 29S (●, ▲), and mock inoculated (○, △). Other explanations about the symbols in the figure were same to Fig. 2.6.

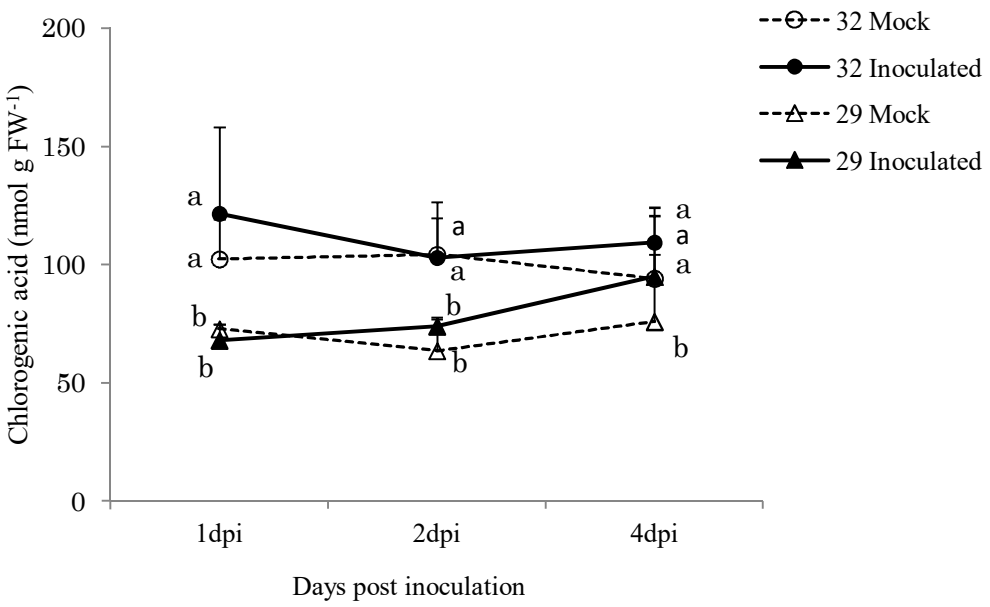

Fig. 2.8. Time course changes of chlorogenic acid (nmol g FW^{-1}) on rice leaves at 1dpi, 2dpi, 4dpi in *R solani* inoculated 32R and 29S (●, ▲), and mock inoculated (○, △). Other explanations about the symbols in the figure were same to Fig. 2.6.

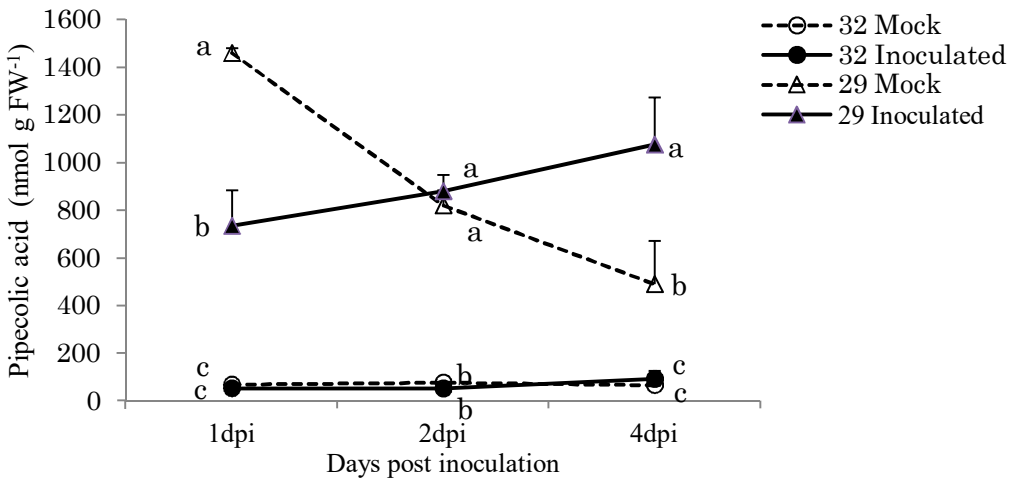

Fig. 2.9. Time course changes of pipecolic acid (nmol g FW^{-1}) on rice leaves at 1dpi, 2dpi, 4dpi in *R solani* inoculated 32R and 29S (●, ▲), and mock inoculated (○, △). Other explanations about the symbols in the figure were same to Fig. 2.6.

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* (Fig. 2.3 and Table 2.1). Chlorogenic acid increased in 32R after inoculation by *R solani* (Fig. 2.2). Based on the time course, the chlorogenic acid level of 32R was higher than 29S (Fig. 2.8). This suggested that chlorogenic acid probably contributed as properties owned by 32R as defense 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 Fig. 2.8, the chlorogenic acid level between mock and inoculated plants in each line was not significantly different, except in 29S at 4 dpi. In addition, the significant difference of chlorogenic acid in inoculated 32R and 29S were detected at 1 dpi. This result suggested two possibilities that chlorogenic acid of 32R was originally higher than that of 29S, and physical injury caused by treatments might appear in both rice lines within 1day after inoculation.

Pipecolic acid in systemic acquired resistance 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 (Fig. 2.3 and Table 2.1). Pipecolic acid was represented in cluster I of hierarchical cluster (Fig. 2.2). 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 (Fig. 2.9). 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 systemic acquired resistance (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 defense mechanism.

Based on the research results by 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 and 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.

4. Summary

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 defense 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 suggests 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 defense 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 1dpi.

Chapter 3

Metabolomic study of two rice lines infected by *Rhizoctonia solani* in negative ion mode by CE/TOF-MS

1. Introduction

Rhizoctonia solani is fungal pathogen causing rice sheath blight disease. Two rice lines, 32R and 29S, were developed from Tetep x CN4-4-2 with different characteristics as response to *R. solani* infection (Wasano et al., 1985). 32R is a resistant to *R. solani*, while 29S is a susceptible to *R. solani* infection. The resistance of rice lines to *R. solani* infection is related to the polygene (Gaihre and Nose, 2011). According to Lindhout (2002), the polygene is designated as quantitative trait loci (QTL). Nine QTL and candidate genes linked with sheath blight resistance in rice have been also identified by Yadav et al (2015). In the nine QTLs, there are two physiological QTLs of β-1,3-glucanase and chitinase, which play a role in the lysis of pathogen cell walls. In 32R, 7 genomic regions related to sheath blight disease resistance were observed by Gaihre et al. (2015). QTL is determined by the position on the genome and the quantitative effect on the resistance. However, QTL cannot adequately explain the mechanism of resistance. Through the present study, resistance mechanisms of rice lines against *R. solani* infection were investigated comprehensively using metabolomic analysis.

In plant resistance, infections by *R. solani* may cause metabolite alterations of biosynthetic pathways (Aliferis and Jabaji, 2012). Previous research found that significant changes in several metabolic enzymes linked with secondary metabolites

occurred in both rice lines (Mutuku and Nose, 2010). In addition, it was also reported that the changes in primary metabolites and enzymatic reactions in the phenylpropanoid and shikimate pathway appeared in infected rice lines (Mutuku and Nose, 2012). This indicates the involvement of primary and secondary metabolites in the plant response to *R. solani* infection.

In a study related to the plant metabolism, Aliferis et al. (2014) reported that the alteration of carbohydrate mobilization and nucleotide metabolism are two main types of primary metabolism involved in plant resistance against *R. solani*. On the other hand, *R. solani* infection was able to induce changes in secondary metabolism, including phenol (Kagale et al., 2011). Phenol compounds are a secondary metabolite group derived from the shikimate-phenylpropanoid-flavonoid pathway (Jimenez-Garcia et al., 2013) and may consist of benzenoid-phenylpropanoid metabolites (Petersen et al., 1999).

Metabolomic analysis by capillary electrophoresis-mass spectrometry (CE-MS) was used for primary metabolite determination in rice leaves (Sato et al., 2004). Furthermore, Sana et al. (2010) applied a similar approach to examine the rice response to the infection of the bacterial blight pathogen. Metabolomic analysis with CE-MS can be conducted by positive and negative ion modes. Each ion mode is utilized for analyzing the cationic and anionic metabolites, respectively (Soga et al., 2003; Soga et al., 2009). In previous research as shown in Chapter 2, metabolomic analysis using CE/TOF-MS in positive ion mode has been conducted to determine amino acid involvement in the resistance mechanism of rice infected by *R. solani* (Suharti et al., 2016). In this study, CE/TOF-MS in negative ion mode was used to comprehensively analyze the metabolites in the rice leaf of 32R and 29S as responses against *R. solani*

infection. This study focuses on the primary metabolites associated with nucleotide and carbohydrate metabolism, as well as secondary metabolites related to phenylpropanoid and benzenoid.

2. Material and Methods

Sample preparation

The rice lines used for the study were 32R and 29S as resistant and susceptible lines to sheath blight disease, respectively (Wasano et al., 1985). The germination, transplantation, and maintenance of rice plant are similar with explanation as shown in Chapter 2.

Pathogen inoculation and sample collection

R. solani was cultured and prepared as shown in Chapter 2. Inoculation method was in accordance with Wasano et al. (1983). Samples were collected in similar leaf position as mentioned in Chapter 2 at 10:00 am of one, two, and four days post-inoculation (dpi). Rapid freezing using liquid nitrogen was used for sample preparation.

CE/TOF-MS analysis in negative ion mode

The samples for CE/TOF-MS were prepared in accordance with Soga et al. (2006) followed by some modifications as explained in Chapter 2. The anion internal standard solution from HMT was used. Sample solution (10 μL) was inserted to the CE vials and injected into CE/TOF-MS in negative mode condition. Cinnamic acid, benzoic acid, vanillic acid, caffeic acid and jasmonic acid (Wako Pure Chemical Industries Ltd, Japan) were prepared each in 1 μg μL^{-1} as external standard.

CE/TOF-MS instrument is similar to those mentioned in Chapter 2. However, it

was arranged for metabolite determination in the negative ion mode. Anion buffer solution (p/n: H3301-1001, HMT) was used as run and rinse buffer. Sheath liquid (p/n: H3301-1020) was provided by HMT. CE and MS capillary voltage were 30 kV and 3500 V, respectively. ESI negative was applied as MS ionization. Drying gas temperature was 300°C.

Raw data were imported to MassHunter (MH) Workstation software on Quantitative Analysis B.06.00 (Agilent Technologies, USA). Data processing was started by extracting of signal peak, continued with normalizing migration time by using the internal standard. Peak alignment was conducted by comparing the value of m/z and normalized migration time of the internal standards. The tolerance range for each value at ± 10 ppm and ±0.5 min was allowed, respectively. Peak area was normalized against those of internal standards to obtain the relative peak area. The data were exported to Mass Profiler Professional (MPP) B.12.60 (Agilent Technologies, USA) for further analysis. To measure metabolite concentration, the resultant peak area was standardized by sample weight.

Statistical Analysis

Filtering, normalization, and baseline z-transform were performed by using MPP software prior to cluster analysis. Further, hierarchical cluster was used to classify the metabolites based on the relative peak area. Volcano plot analysis was carried out based on p-value ($-\log_{10}$) and fold change ($\log_2$) of the metabolic data in comparison of two rice lines in *R. solani* infection and mock treatment. Time course change data were analyzed using Tukey's test.

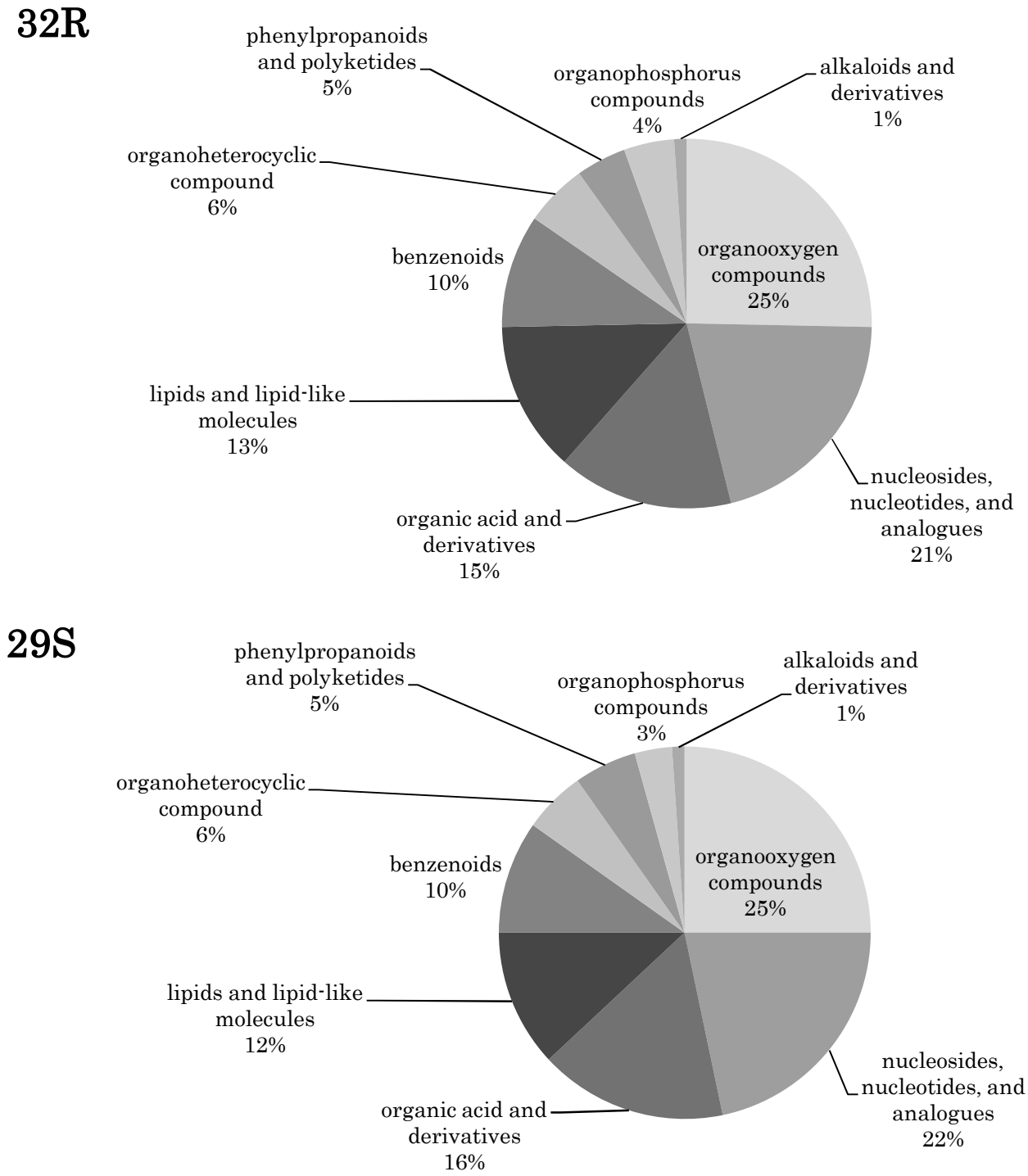

Fig. 3.1. Compound grouping of metabolites on 32R and 29S under *R. solani* and mock treatment based on super-class of chemical taxonomy based on public database.

Table 3.1. Chemical taxonomy of 44 metabolites from hierarchical cluster based on public database

No.	Metabolite	Super class	Class	Sub class
1.	Glyceric acid	organooxygen compounds	carbohydrates and carbohydrate conjugates	sugar acid and derivatives
2.	cGMP	nucleosides, nucleotides, and analogues	purine nucleotides	purine ribonucleotides
3.	IAA	organoheterocyclic compound	indole and derivatives	indolyl carboxylic acid and derivatives
4.	ADP	nucleosides, nucleotides, and analogues	purine nucleotides	purine ribonucleotides
5.	Jasmonic acid	lipids and lipid-like molecules	lineolic acids and derivatives	jasmonic acid
6.	UDP galactose	nucleosides, nucleotides, and analogues	pyrimidine nucleotides	pyrimidine nucleotide sugars
7.	Glucaric acid	organooxygen compounds	carbohydrates and carbohydrate conjugates	sugar acid and derivatives
8.	Pimelic acid	lipids and lipid-like molecules	fatty acyls	fatty acids and conjugates
9.	*N*-acetylglucosamine 6P	organooxygen compounds	carbohydrates and carbohydrate conjugates	aminosaccharides
10.	UDP	nucleosides, nucleotides, and analogues	pyrimidine nucleotides	pyrimidine ribunucleotides
11.	3-Hydroxypropionic	organic acid and derivatives	hydroxy acids and derivatives	beta hydroxy acids and derivatives
12.	Benzoic acid	benzenoids	benzene and substituted derivatives	benzoic acid and derivatives
13.	Shikimic acid	organooxygen compounds	alcohol and polyols	cyclic alcohols and derivatives
14.	2-Phosphoglyceric acid	organooxygen compounds	carbohydrates and carbohydrateconjugates	sugar acid and derivatives
15.	Uric acid	alkaloids and derivatives		
16.	Vanillic acid	benzenoids	benzene and substituted derivatives	benzoic acid and derivatives
17.	Trehalose 6P	organooxygen compounds	carbohydrates and carbohydrate conjugates	dissacharides
18.	Glucosamine 6P	organooxygen compounds	carbohydrates and carbohydrate conjugates	monosaccharides
19.	Dihydroxyacetone phosphate	organooxygen compounds	carbonyl compounds	ketones
20.	Glyceraldehyde 3P	organooxygen compounds	carbohydrates and carbohydrate conjugates	monosaccharides
21.	dUMP	nucleosides, nucleotides, and analogues	pyrimidine nucleotides	pyrimidine deoxyribonucleotides
22.	2-Oxoglutaric acid	organic acid and derivatives	keto acids and derivatives	gamma-keto acids and derivatives
23.	AMP	nucleosides, nucleotides, and analogues	purine nucleotides	purine ribonucleotides
24.	UMP	nucleosides, nucleotides, and analogues	pyrimidine nucleotides	pyrimidine ribonucleotides
25.	2,5-Dihydroxybenzoic acid	benzenoids	benzene and substituted derivatives	benzoic acid and derivatives
26.	Salicylic acid	benzenoids	benzene and substituted derivatives	benzoic acid and derivatives
27.	*p*-Hydroxybenzoic acid	benzenoids	benzene and substituted derivatives	benzoic acid and derivatives
28.	Lactic acid	organic acid and derivatives	hydroxy acids and derivatives	fatty acyl thioesters
29.	2-Aminoethylphosphonic acid	organophosphorus compounds	organic phosponic acids and derivatives	organic phosphonic acids
30.	AICAR	nucleosides, nucleotides, and analogues	imidazole ribonucleosides and ribonucleotides	1-phosphoribosyl-imidazoles
31.	Sedoheptulose 7P	organooxygen compounds	carbohydrates and carbohydrate conjugates	glycosyl compounds
32.	Mucic acid	organooxygen compounds	carbohydrates and carbohydrate conjugates	sugar acid and derivatives
33.	IMP	nucleosides, nucleotides, and analogues	purine nucleotides	purine ribonucleotides
34.	Isobutyric acid	organic acid and derivatives	carboxylic acid and derivatives	carboxylic acid
35.	CTP	nucleosides, nucleotides, and analogues	pyrimidine nucleotides	pyrimidine ribonucleotides
36.	TTP	nucleosides, nucleotides, and analogues	pyrimidine nucleotides	pyrimidine ribonucleotides
37.	Fumaric acid	organic acid and derivatives	carboxylic acid and derivatives	dicarboxylic acids and derivatives

No.	Metabolite	Super class	Class	Sub class
38.	Gluconic acid	organooxygen compounds	carbohydrates and carbohydrate conjugates	sugar acid and derivatives
39.	Glucose 1P	organooxygen compounds	carbohydrates and carbohydrate conjugates	monosaccharides
40.	Glucose 6P	organooxygen compounds	carbohydrates and carbohydrate conjugates	monosaccharides
41.	Caffeic acid	phenylpropanoids and polyketides	cinnamic acid and derivatives	hydroxycinnamic acids and derivatives
42.	Cinnamic acid	phenylpropanoids and polyketides	cinnamic acid and derivatives	cinnamic acid
43.	Fructose 6P	organophosphorus compounds	organic phosponic acids and derivatives	phospate ester
44.	Isocitric acid	organic acid and derivatives	carboxylic acid and derivatives	tricarboxylic acid and derivatives

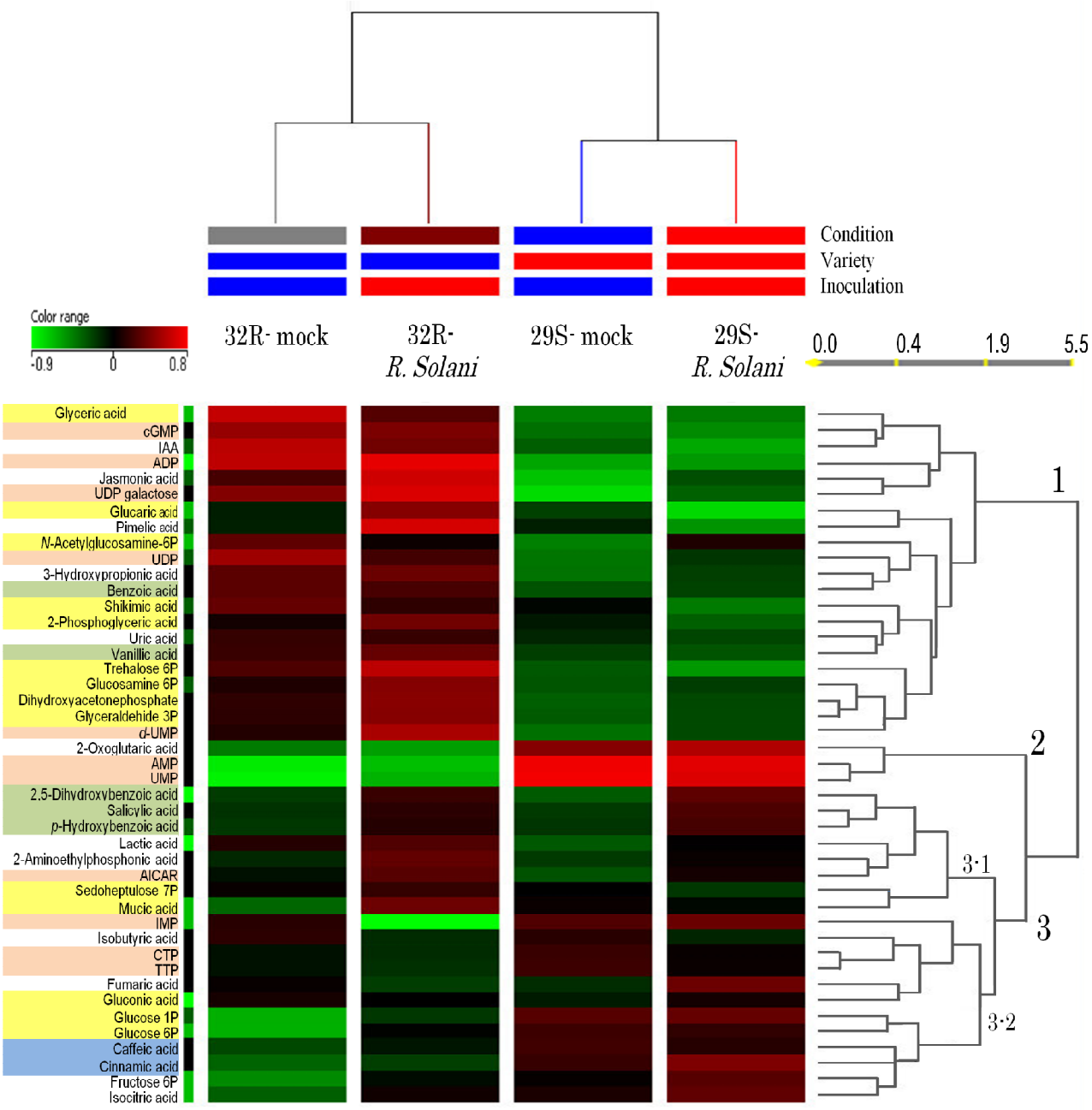

Fig. 3.2. Hierarchical cluster of 44 metabolites based on *Euclidean distance* after filtered by frequency using MPP analysis. In the figures, red and green colors as color range representup-regulation and down-regulation, respectively. Organooxygen compounds showed in yellow border. Nucleosides, nucleotides, and analogues super-class displayed in orange border. Benzenoids and phenylpropanoid super class presented in green and blue border, respectively.

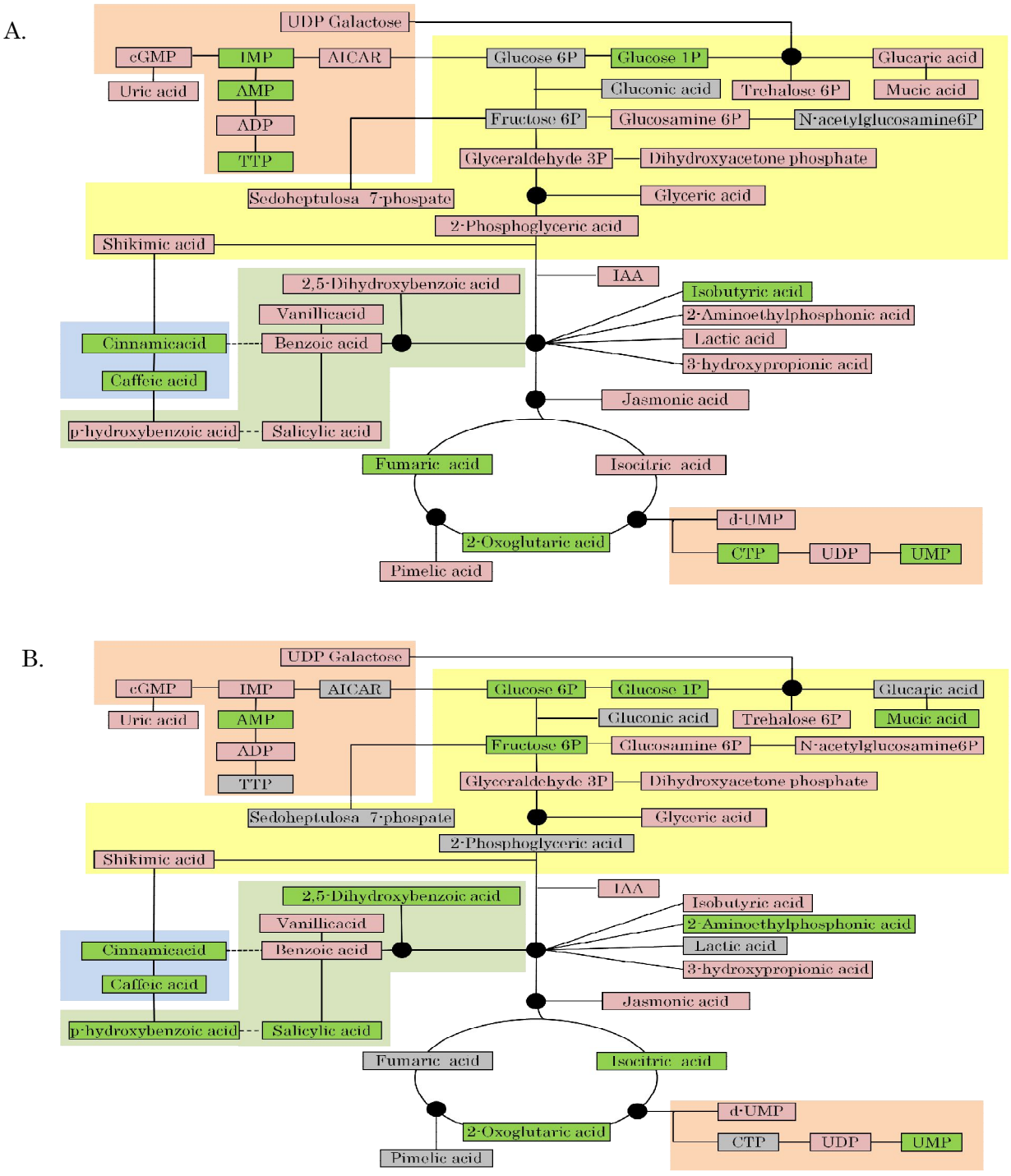

Fig. 3.3. Distribution of related metabolites on primary and secondary metabolite levels of 32R at average of 1dpi, 2dpi, and 4dpi, based on hierarchical cluster data (Figure2). A; 32R infected by *R. solani*, B; 32R mock treatment. The increase and decrease intensity of metabolites was shown in red and green color in metabolite box. Organooxygen compounds were presented in yellow border. Nucleosides, nucleotides, and analogues super-class were displayed in orange border. Benzenoids and phenylpropanoid super class were presented in green and blue border, respectively.

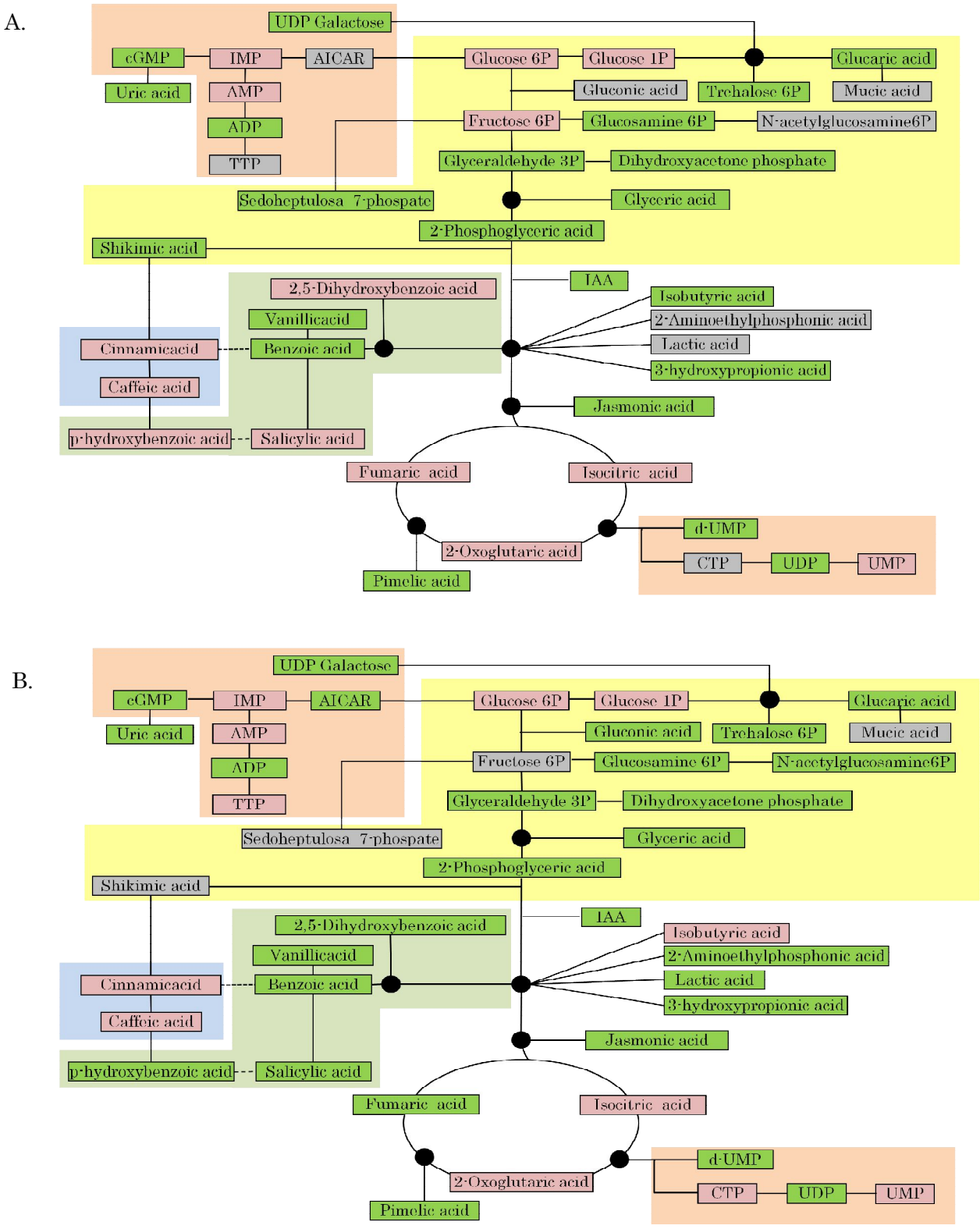

Fig. 3.4. Distribution of related metabolites on primary and secondary metabolite levels of 29S at average of 1dpi, 2dpi, and 4dpi, based on hierarchical cluster data (Figure 2). A; 29S infected by *R. solani*, B; 29S mock treatment. Other explanations in the figure were similar with Fig. 3.3.

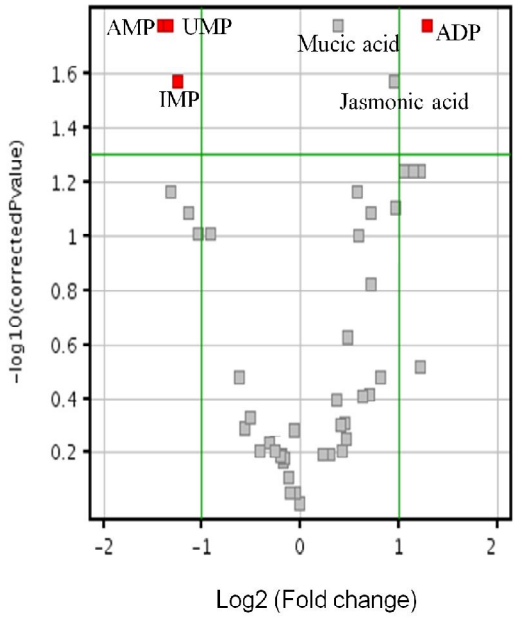

Fig. 3.5. Volcano plot analysis of identified metabolite between 32R and 29S rice lines after inoculated by *R. solani* at 1dpi, 2dpi, and 4dpi in average. Red square represents the metabolite displayed with larger magnitude fold-changes (x-axis, FC≥2.0) and statistical significance difference (y-axis, P≤0.05).

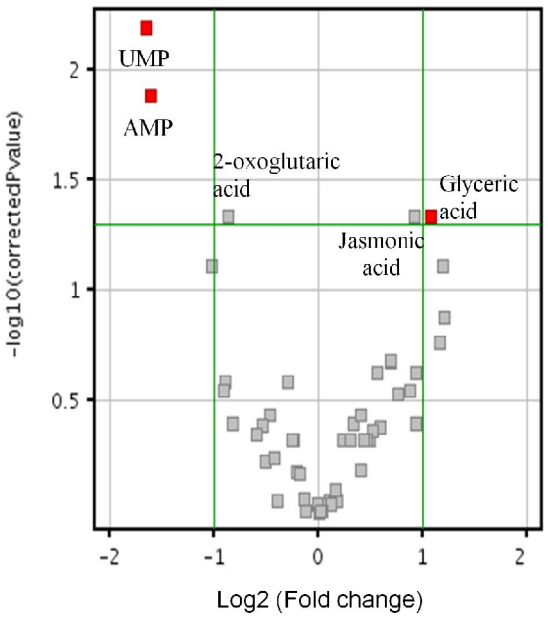

Fig. 3.6. Volcano plot analysis of identified metabolite between 32R and 29S rice lines mock treatment at 1dpi, 2dpi, and 4dpi in average. Other explanations in the figure were similar with Fig. 3.5.

Table 3.2. Up-down regulation metabolite and abundance in volcano plot analysis of resistant (32R) and susceptible (29S) rice lines after inoculated by *R. solani*

No.	Compound	p	p (Corr)	$-\log_{10}$ (corrected p value)	FC (abs)	FC	$\log_2$ (FC)
1.	adenosine monophosphate (AMP)	7.03E-04	0.016391	1.7853945	2.65808	-2.65808	1.4103845
2.	uridine monophosphate (UMP)	9.28E-04	0.016391	1.7853945	2.543595	-2.54356	1.346869
3.	inosine monophosphate (IMP)	0.00303	0.026768	1.5723841	2.377973	-2.37797	1.2497323
4.	adenosine diphosphate (ADP)	9.01E-04	0.016391	1.7853945	2.440266	2.44026	1.2870384

Corr; corrected, FC; fold change.

Table 3.3. Up-down regulation metabolite and abundance in volcano plot analysis of resistant (32R) and susceptible (29S) rice lines in mock treatment

No.	Compound	p	p (Corr)	$-\log_{10}$ (corrected p value)	FC (abs)	FC	$\log_2$ (FC)
1.	uridine monophosphate (UMP)	1.74E-04	0.006414	2.192871	3.138378	-3.13838	1.650019
2.	adenosine monophosphate (AMP)	7.20E-04	0.012954	1.887596	3.061395	-3.06140	1.614189
3.	glyceric acid	0.00357	0.045946	1.337752	2.117993	2.117993	1.082698

Corr; corrected, FC; fold change.

3. Results and Discussion

General CE/TOF-MS analysis in negative ion mode

By using CE/TOF-MS, 110 metabolites were successfully identified in both rice lines by Masshunter (MH) software. Based on the chemical taxonomy for the detected metabolites, the percentage of each group in the 32R and 29S under *R. solani* and mock treatment were similar (Fig. 3.1 and Table 3.1). Two groups appeared as the largest percentage groups: organooxygen compounds, and nucleosides, nucleotides and analogues. The percentage of organooxygen group was 25%. Furthermore, 21% of 32R and 22% of 29S were detected as nucleosides, nucleotides, and analogues super-class. In this study, metabolites were grouped in the organooxygen compound superclass dominated by sugar and derivatives, and monosaccharides sub-class (Table 3.1). As explained by Soga et al. (2003) and Ahmad (2013), analysis negative ion mode is applicable for anionic metabolites such as sugar phosphate, organic acid, CoA compounds, and nucleotides.

Previous research as shown in Chapter 2 demonstrated that most metabolites detected by positive ion mode were grouped in amino acid, peptides and analogues sub-class as members of organooxygen compound super-class (Suharti et al., 2016). Furthermore, all metabolites detected were included in cationic metabolite. As explained by Ahmad (2013), the analysis of cationic metabolites is appropriate for amino acid, phenolic, carboxylic, thiol, and phosphate/sulfate group. The result of previous research in Chapter 2 indicates the involvement of phenolic compound and amino acid metabolites in plant resistance and susceptibility due to *R. solani* infection, respectively (Suharti et al., 2016).

In this study, the metabolites were analyzed using MPP software. Detected

metabolites were filtered by frequency to create higher quality data. Filter by frequency was conducted to remove irreproducible metabolites. It reduced the metabolites from 110 into 44 metabolites based on appearance with a frequency of 100%. The analysis was followed by hierarchical cluster analysis based on Euclidean distance (Fig. 3.2). The high and low levels of metabolites were represented by red and green color, respectively. At the 50% level of dissimilarity (at 2.75 of Euclidean distance), the metabolites were divided into 3 clusters. The large grouping of cluster was observed in the first cluster, which included 21 metabolites. The first cluster showed the high intensity level of metabolites in 32R. In contrast, classification in cluster 2 showed high metabolites level of 29S. Cluster 3 consisted of 2 sub-clusters. One sub cluster showed the domination of high metabolite levels in 32R and 29S inoculated by *R. solani*. Another sub-cluster showed lower metabolite levels in 32R inoculated by *R. solani.*

The metabolic map in Figs. 3.3 and 3.4 shows the changes in metabolites based on hierarchical cluster. Metabolic map were dominated by the primary metabolite, particularly organooxygen compound followed by nucleosides, nucleotides, and analogues super-class. The secondary metabolites in metabolic map were grouped in phenylpropanoid and benzenoids super class.

The metabolites were further filtered by volcano plot analysis using MPP software. Forty-four metabolites were filtered based on p-value ($-\log_{10}$) versus fold change ($\log_2$) on the y and x-axes, respectively. The metabolites were cut off based on p-value ≤ 0.05 and fold change ≥ 2.0 in the interaction of rice lines and inoculation. Volcano plot in the comparison between 32R and 29S–*R. solani* inoculated showed 4 metabolites with significant p-value and high fold change (Fig. 3.5 and Table 3.2). ADP was up-regulated in 32R relative to 29S-*R. solani* inoculated. AMP, UMP and IMP were

down-regulated in 32R relative to 29S-*R. solani* inoculated. The comparison between 32R and 29S-mock treatment based on volcano plot is shown in Fig. 3.6 and Table 3.3 Glyceric acid showed up-regulated in 32R compared to 29S-mock treatment. Meanwhile, UMP and AMP showed down-regulated in 32R-mock treatment relative to 29S-mock treatment. Subsequently, each metabolite was assessed based on time course as shown in Fig. 3.7. A detailed discussion will follow in the next section.

Primary metabolites in rice lines infected by R. solani

Nucleotides metabolism in plant pathogen interaction.

The involvement of nucleosides, nucleotides, and analogues group in the interaction of rice line and *R. solani* infection was observed by using volcano plot analysis (Fig. 3.5). ADP showed abundant in 32R infected by *R. solani* relative to 29S in the same treatment. The abundance of ADP may be associated with the high respiration in plants due to pathogen infection (Agrios, 1988), which provide the energy for the synthesis of defense compounds in the host plant (Morkunas et al., 2013). In order to clarify the volcano plot results, the content of ADP was evaluated by time course change (Fig. 3.7). ADP showed increases in every treatment, but those in 32R-*R. solani* inoculated were significantly higher during 1 to 4dpi. Based on this analysis, it is suggested that the high respiration has occurred in the 32R-*R. solani* inoculated. ADP converts to ATP to provide energy in plant respiration of the diseased plant (Millerd and Scott, 1962). Unfortunately, ATP was not detected in this research. It is likely that the use of a stainless steel ESI sprayer needle influenced the ATP detection in the negative mode as reported by Soga et al. (2009).

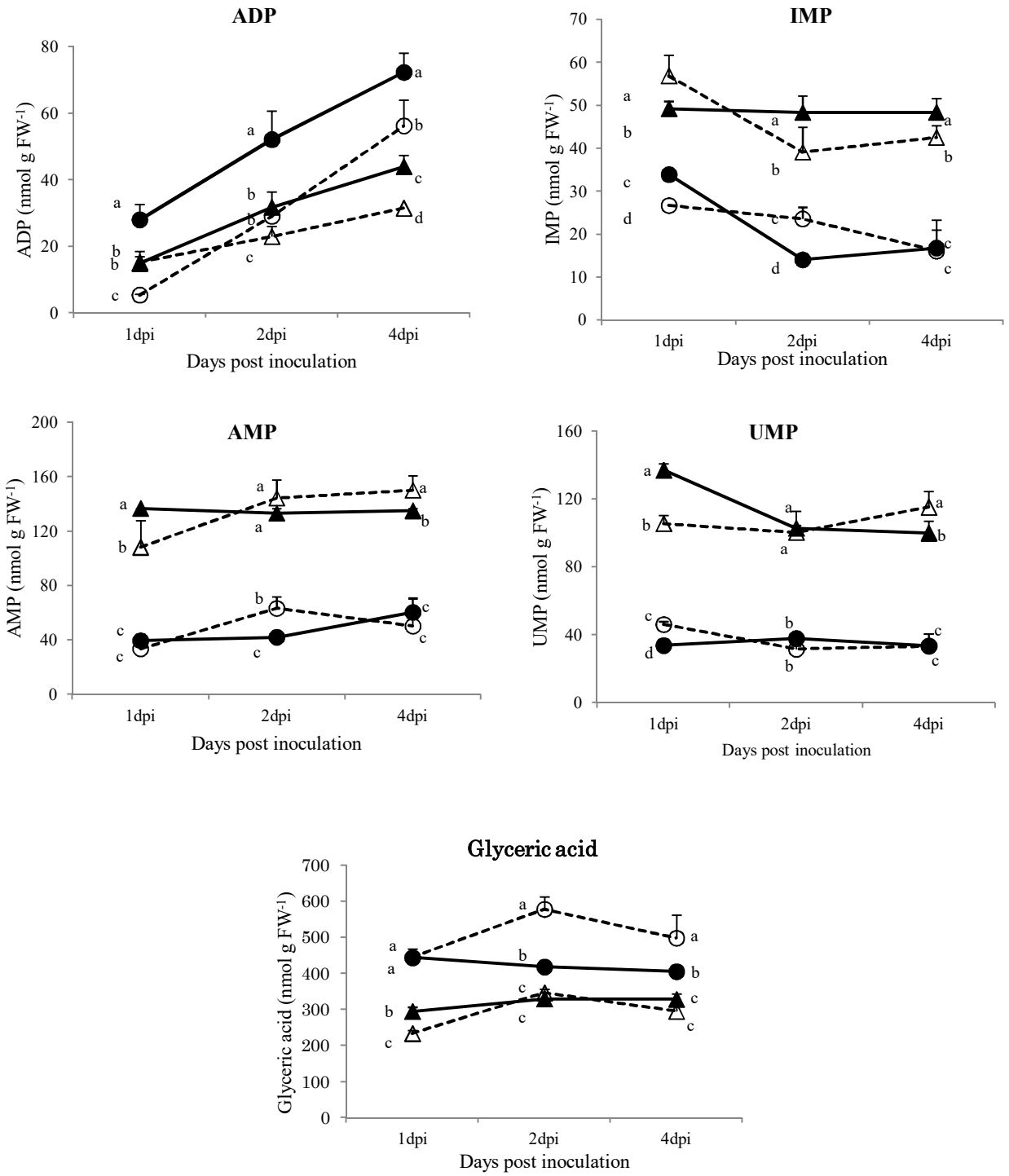

Fig. 3.7. Time course changes of up-down regulated metabolite(nmol g FW^{-1}) based on volcano plot on rice leaves at 1dpi, 2dpi, 4dpi in *R solani* inoculated of 32R and 29S (●, ▲), and mock treatment of 32R and 29S (○, △). For each time course, values followed by the same letter are not statistically different ($p<0.05$) by one-way ANOVA test followed by tukey's test hsd (honestly significant different) at each dpi.

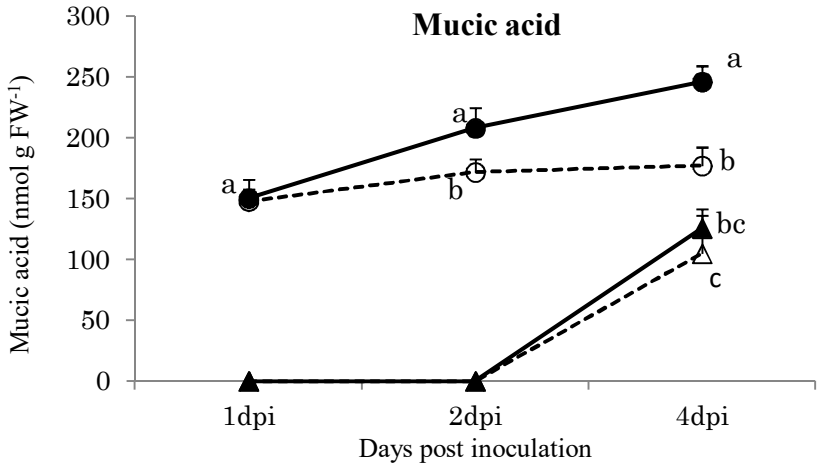

Fig. 3.8. Time course changes of mucic acid (nmol g FW^{-1})on rice leaves at 1dpi, 2dpi, 4dpi in *R solani* inoculated of 32R and 29S (●, ▲), and mock treatment of 32R and 29S (○, △). Other explanations about the symbols in the figure were similar with Fig. 3.7.

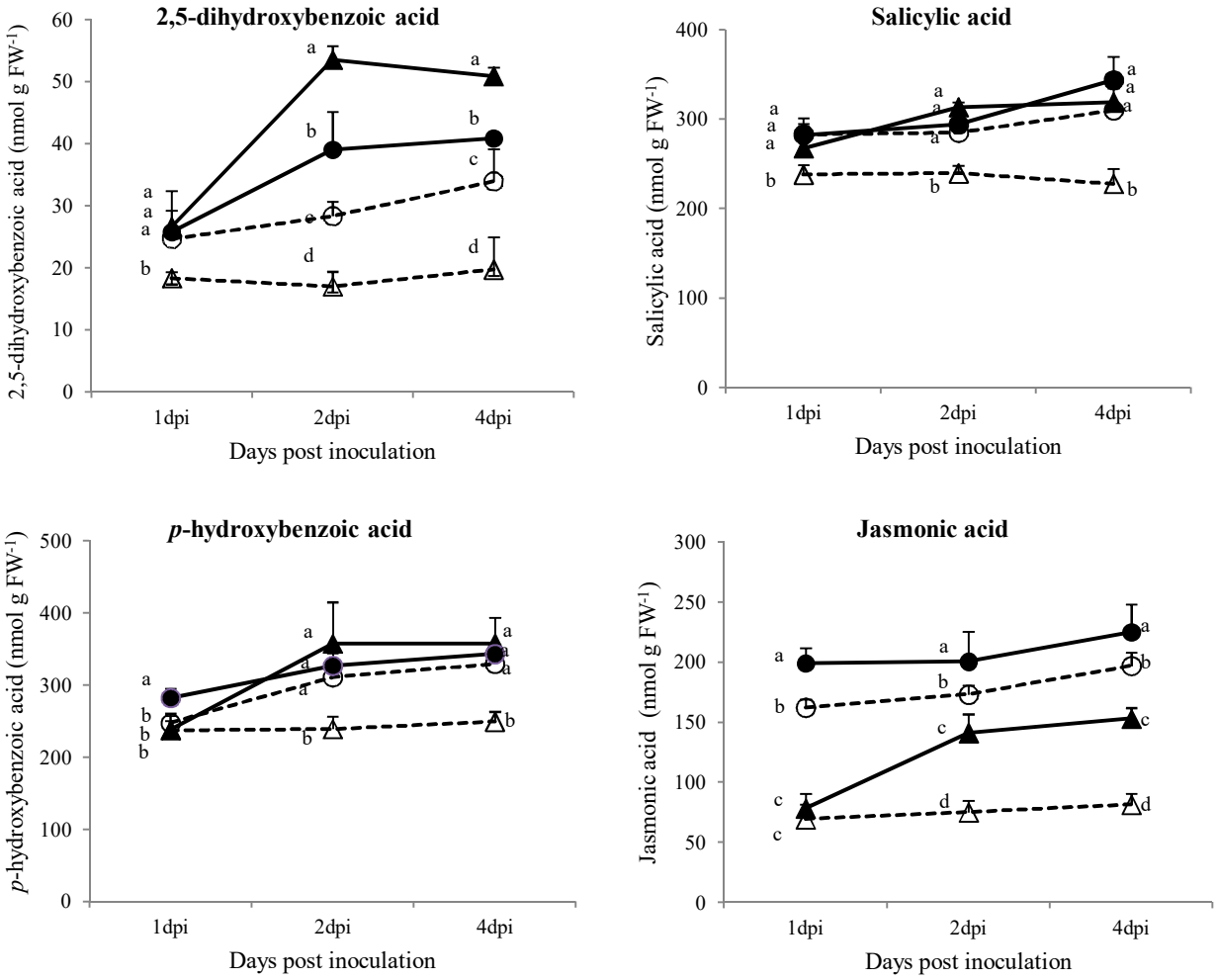

Fig. 3.9. Time course changes of secondary metabolite (nmol g FW^{-1}) on rice leaves at 1dpi, 2dpi, 4dpi in *R solani* inoculated of 32R and 29S (●, ▲), and mock treatment of 32R and 29S (○, △). Other explanations about the symbols in the figure were similar with Fig. 3.7.

IMP abundance in 29S inoculated by *R. solani* was observed by volcano plot analysis (Fig. 3.5). Based on the time course change in Fig. 3.7, IMP content in the 32R was lower than in 29S. IMP in *R. solani* and mock treatment of both rice lines tended to decrease during 1 to 2dpi. IMP in 29S-*R. solani* inoculated showed a stable and high tendency during 2 to 4dpi. This suggests that inoculation of *R. solani* in 29S induced the IMP content. According to Smith and Atkins (2002), IMP is associated with the enhancement of nitrogen assimilation in plant. It is strongly related to amino acid accumulation (Lawlor, 2002), which may promote the disease severity in plant (Tavernier et al., 2007). Amino acid is derived from glycolysis, pentose phosphate pathway or TCA pathway (Berg et al., 2002). Metabolites involved in amino acid metabolism mapped in those pathways showed an increase in 29S infected by *R. solani* compared to 32R (Fig. 3.3 and 3.4). IMP in this study is related to the previous findings in sheath blight disease resistance gene. Gaihre et al. (2015) successfully encoded the cytokinin *O*-glucosyltransferase in chromosome 7 as QTL for sheath blight disease resistance in 32R. The cytokinin *O*-glucosyltransferase was catalyzed in the conjugation between cytokinin and *O*-glucoside (Rodó et al., 2008). Cytokinin is known as the nitrogen metabolism proponent in plants (Igarashi et al., 2008; Takei et al., 2002). In previous research as shown in Chapter 2, 32R had lower amino acids than 29S (Suharti et al., 2016). This evidence is also supported by IMP in this study. It is likely that the nitrogen metabolism in 32R was depressed due to *R. solani* infection. Thus, QTL of the cytokinin *O*-glucosyltransferase found in 32R as sheath blight disease resistance may play a negative effect to the amino acid accumulation.

Analysis by a volcano plot in Fig. 3.5 shows the down regulation of AMP and UMP in 32R relative to 29S-*R. solani* inoculated. On the other hand, these metabolites

also showed down regulation in 32R relative to 29S in mock treatment (Fig. 3.6). AMP and UMP are ribonucleotide as RNA structural unit. AMP was involved in IMP biosynthesis which is the first key intermediate in purine base biosynthesis (Kappock et al., 2000), while the UMP was involved in the metabolic pathway of pyrimidine (Bogan and Brenner, 2010). The abundance of AMP and UMP in 29S showed the possibility of negative expression in this phenomenon. Subsequently, the abundance of both metabolites in the 29S suggested that the *R. solani* may not induce the activity of these metabolites. Based on the time course change (Fig. 3.7), AMP and UMP of 29S tend to be stable and high during 1 to 4dpi. This suggested that the possibility of injury from artificial inoculation treatment may affect the results. In the future, the roles of AMP and UMP need to be confirmed because of their importance in the process of plant physiology.

Carbohydrate metabolism involvement in plant defense mechanism

The involvement of carbohydrate metabolism in the defense mechanism of rice lines against *R. solani* was observed simultaneously through hierarchical cluster analysis. The first cluster demonstrated the high level of metabolite of 32R relative to 29S in both *R. solani* and the mock treatment (Fig. 3.2). Metabolites in the first cluster were dominated by the organooxygen compound consisting of carbohydrate and conjugates (Table 3.1). Those metabolites, glyceric acid, glucaric acid, *N*-acetyglucosamine, 2-phosphoglyceric acid, trehalose 6P, glucosamine 6P, and glyceraldehide 3P are involved in carbohydrate metabolism. The mentioned metabolites were changed due to *R. solani* infection in 32R. The carbohydrate metabolite alteration is one of plant responses against pathogen infection (Bolton, 2009). The enhancement of

carbohydrate metabolism induces an increase in the sugar content in plants. Sugar accumulation plays a role in plant immune system and plant defense signaling due to the presence of pathogens (Morkunas and Ratajczak, 2014). Conversely, the reduction of carbohydrate availability in plants promotes the plant susceptibility to pathogen infection (Engelsdorf et al., 2013).

The relationship between carbohydrates and respiration processes through glycolysis and the TCA cycle was observed by metabolic mapping (Figs. 3.3 and 3.4). Four metabolites, glucose 6P, fructose 6P, glyceradehide 3P, and 2-phosphoglyceric acid are involved in the glycolysis pathway. Meanwhile, isocitric acid, 2-oxoglutaric acid, and fumaric acid are involved in the TCA cycle. Based on the metabolic map (Fig. 3.3), 2-phosphoglyceric acid increased due to *R. solani* infection. In the 29S, the infection of *R. solani* increased the level of fructose 6P (Fig. 3.4). In the TCA cycle, *R. solani* infection increased the isocitric acid level in 32R (Fig. 3.3). Meanwhile, the fumaric acid level increased in 29S after infection by *R. solani* (Fig. 3.4). The increase in the metabolite level in glycolysis and the TCA cycle indicates the enhancement of respiration in both rice lines after *R. solani* infection. The increase in respiration due to *R. solani* infection has been observed by Agarwal (2010). These results also agree with the idea that 32R may have a higher respiration than 29S based on the accumulation of ADP after infection. According to Fernie et al. (2004), respiration consists of three main pathways: glycolysis, the TCA cycle and mitochondrial electron transport. A previous study in our lab showed the enzyme activity related to glycolysis in 32R and 29S infected by *R. solani* (Mutuku and Nose, 2012). The activity of phosphofructokinase (PFK), triosephosphate isomerase (TPI), phosphoglycerate kinase (PGK), enolase and pyruvate kinase (PK) in glycolytic pathway increased in plants infected by *R. solani*. In

addition, Mutuku and Nose (2010) showed the increased expression of glycolytic enzymes associated with the activation of the TCA cycle. This result indirectly explains that high respiration occurs in 32R infected by *R. solani*.

Analysis using a volcano plot in Fig. 3.6 shows up-regulation of glyceric acid in 32R relative to 29S-mock treatment. Based on time course change, the glyceric acid in 32R was stable and high during 1 to 4dpi (Fig. 3.7). Meanwhile, glyceric acid in 29S was lower than 32R. Glyceric acid in 32R may reveal the occurrence of photorespiration as explained by Peterhansel et al. (2010), and can regulate plant defense to biotic stress (Kangasjärvi et al., 2012). The photorespiration pathway is used to prevent excess reactive oxygen species (ROS) accumulation. Photorespiration can minimize ROS accumulation by dissipating excess reducing equivalents and energy, such as regeneration of ADP from ATP consumption to convert glyceric acid to phosphoglyceric acid (Voss et al., 2013). The glyceric acid in 29S may correlate with ROS stimulation in the plant, as explained by Suzuki et al. (2013). The ROS will trigger the emergence of cell-death and increase the susceptibility of plants if the ROS exceeds the threshold (Barna et al., 2012).

The involvement of carbohydrate metabolism in the defense mechanism against *R. solani* was also observed in cluster 3 sub cluster 1. Mucic acid increased in 32R due to *R. solani* infection (Fig. 3.2). Mucic acid is an important constituent metabolite of pectin synthesis (Richard and Hilditch, 2009). Based on the volcano plot (Fig. 3.5), mucic acid showed abundance in 32R-*R. solani* inoculated, with a higher p-value but low fold change. The increase of mucic acid in 32R infected by *R solani* is one of the plant's defenses that may be associated with the formation of pectin. Pectin is the primary cell wall constituent compound as a plant barrier against the invasion of

pathogens (Malinovsky et al., 2014). The induction of pectin as a plant response to pathogen infection is a structural defense mechanism in the primary cell wall (Pogorelko et al., 2013). Based on the time course change, mucic acid was showed to be high and stable in 32R during 1 to 4dpi (Fig. 3.8). This suggests that mucic acid may act as a defense mechanism in the 32R due to *R. solani* infection. Previous research in our laboratory reported that lignin as a secondary cell wall was accumulated in 32R after *R. solani* infection (Mutuku and Nose, 2012). Similarly, Danson (1999) reported that lignin accumulation plays a role as the first line of defense of resistant rice plant to *R. solani* infection. Accumulation of mucic acid in 32R due to the infection suggests that *R. solani* may not only induce the secondary cell wall as a defense mechanism, but also promote the primary cell wall.

Secondary metabolites in rice lines infected by R. solani

Phenylpropanoid and benzenoid involvement in plant defense due to R. solani infection

Phenylpropanoid and benzenoid as secondary metabolites were observed in this study. Based on the hierarchical cluster (Fig. 3.2), caffeic acid and cinnamic acid as phenylpropanoid metabolites were included in cluster 3 sub cluster 2. Both metabolites play a role as a precursor of lignin formation in plant defense (Bubna et al., 2011; Salvador et al., 2013). According to Mutuku and Nose (2012), lignin was accumulated at the inoculation site of both rice lines promoted by phenylalanine ammonia lyase (PAL) activation. The lignin deposition also occurs on the leaves of rice, which are distant from the inoculation site (Danson, 1999).

As shown in the hierarchical cluster (Fig. 3.2), benzenoid metabolites were

distributed in cluster 1 and cluster 3. Benzoic acid and vanillic acid were included in cluster 1. Other benzenoid metabolites; 2,5-dihydroxybenzoic acid, salicylic acid, and *p*-hydroxybenzoic acid were grouped in cluster 3 sub cluster 1. As shown in the metabolic map, the level of 2,5 dihydroxybenzoic acid, salicylic acid, and *p*-hydroxybenzoic acid increased in both rice lines after *R. solani* infection (Figs. 3.3 and 3.4). Based on the time course change (Fig. 3.9), 2,5-dihydroxybenzoic acid increased sharply in 32R and 29S after 2dpi. According to Dempsey et al. (2011), the accumulation of 2,5-dihydroxybenzoic acid is related to salicylic acid as plant response to pathogen infection through hydroxylation. It is also reported by Bartsch et al. (2010) that 2,5-dihydroxybenzoic acid is associated with salicylic acid oxidation by ROS. In the same Figure, a significant increase of salicylic acid in 29S occurred early after *R. solani* infection along with the time observation since 1 dpi to 4dpi. This occurrence may be related to the role of salicylic acid as the fundamental defense system in rice (Silverman et al., 1995). In 32R, *p*-hydroxybenzoic acid increased at 1dpi. Meanwhile in 29S, *p*-hydroxybenzoic acid increased at 2dpi and 4dpi. Based on the time course change, those secondary metabolites increased in 32R and 29S due to *R. solani* infection. The increase of *p*-hydroxybenzoic acid level triggered the programmed cell death in plant due to pathogen infection as a defense mechanism in resistant plants (Veit, et al., 2001). In addition, *p*-hydroxybenzoic acid can inhibit the growth of *R. solani* (Siddiqui and Shaukat, 2002).

Jasmonic acid role as plant signaling in rice lines infected by R. solani

In this study, jasmonic acid was detected as an important plant signaling metabolite in plant defense mechanism against fungal pathogen. Jasmonic acid is a

secondary metabolite from lipids and lipid-like molecules super class (Table 3.1). Based on the hierarchical cluster and metabolic map (Figs. 3.2, 3.5 and 3.6), jasmonic acid increased in 32R compared to 29S. By volcano plot analysis (Fig. 3.3), jasmonic acid showed abundance in 32R infected by *R. solani* with a high p-value.

Based on the time course change (Fig. 3.9), 32R and 29S showed different responses due to *R. solani* infection. Jasmonic acid was stable and high in 32R during 1 to 4dpi. Jasmonic acid in 32R infected by *R. solani* was higher than 32R-mock treatment. Jasmonic acid is one plant responses against the infection of necroptrophic pathogens (Mengiste, 2012). Jasmonic acid can induce the resistant of the plant during pathogen infection by promoting the lignifications as a physical barrier in the defense mechanism against *R. solani* (Taheri and Tarighi, 2010). As shown in Fig. 3.9, jasmonic acid in 29S was lower than 32R. According to Mutuku (2012), lignifications occurred in both rice lines at 1dpi due to *R. solani* infection with different amount of lignin deposition. The lignification appeared obviously in 32R, while it showed very low accumulation in 29S (Danson, 1999). Based on these results, it was suggested that a delay of jasmonic acid accumulation in 29S results in low lignification as a plant response to the *R. solani* infection.

4. Summary

We used CE/TOF-MS in negative ion mode to successfully investigate the primary and secondary metabolites in two rice lines under *R. solani* infection. As a result, a comprehensive metabolite profiling analysis has been established. The investigation of primary and secondary metabolites has revealed different responses between the rice lines (32R and 29S, respectively) in plant defense against *R. solani*

infection. The inoculation of *R. solani* induced an increase in metabolites as a specific response of each rice line. *R. solani* infection led to the increase of ADP, glyceric acid, mucic acid and jasmonic acid in the 32R. The increase of ADP level indicated the enhancement of respiration to obtain energy for plant defense mechanism. Glyceric acid abundance in the 32R was associated with photorespiration. The mucic acid in this study indicated the influence of *R. solani* to promote the primary cell wall formation as a defense mechanism. The increased of jasmonic acid may be associated with lignin accumulation as defense mechanism in secondary cell wall deposition. In 29S, IMP increased after *R. solani* infection. It may be associated with nitrogen mobilization which promotes the disease severity. IMP also confirms the previous result in QTL and amino acid. The increase of phenylpropanoid and benzenoid compound in both rice lines may show the response of rice lines to *R. solani* infection.

This study is fundamental research related to several important metabolisms in the plant pathogen interaction. Therefore, in the future, confirmation of the significant results obtained in this study, such as the deposition of pectin, respiration, and the signaling system will be performed using the same rice lines under *R. solani* infection.

Chapter 4

Canavanine involvement in the interaction of rice lines and *Rhizoctonia solani*

1. Introduction

Canavanine is metabolite with structural analogues of arginine which involved in the ornithine-urea cycle (Rosenthal, 1982). The determination of canavanine is rarely to be discussed. Previously, colorimetric method, qualitative micro-thin-layer chromatography method and high-performance liquid chromatography (HPLC) were used to determine the canavanine concentration in plant tissue (Archibald 1946; Oropeza et al., 1988; Rajkowksi, 2004). In our previous study in Chapter 2 (Suharti et al., 2016), although the canavanine was detected in both rice lines due to *R. solani* infection, the contents of canavanine in several samples were not stable. At this time, by using MassHunter Quantitative analysis could overcome successfully the obstacle.

Canavanine is known as plant growth inhibitors (Nakajima et al., 2001, Krasuska et al., 2016). In addition, canavanine plays a role as potential antifungal and antibacterial metabolite against plant pathogen (Wink, 1988). According to Simola and Lönnroth (1979), canavanine has effects as pathogen growth inhibitor. This occurrence reinforces the suggestion that canavanine may play a role in plants defense against pathogen infection. Through this study, canavanine can be identified in rice plants and proposed to be involved in the defense mechanism of rice lines against *R. solani* infection.

2. Materials and Methods

Rice seed from 32R and 29S were germinated and maintenanced as explained in Chapter 2. The pathogen preparation and inoculation was done according to Wasano et al. (1983). Sample preparation for CE/TOF-MS was carried out as described by Suharti et al. (2016) as shown in Chapter 2. Capillary electrophoresis (Agilent 7100 CE, Agilent Technologies, USA) with Agilent G6224A time- of- flight -mass spectrometry (CE/TOF-MS) was used in the positive ion mode. An Agilent 1100 isocratic high-performance liquid chromatography (HPLC) pump, an Agilent G1603A CE-MS adapter, CE-ESI-MS sprayer kit (G1607A-60041), and fused silica capillary (50 μm i.d. x 80 cm total length, HMT, Japan) were used in this study. Cation buffer solution (p/n: H3301-1001) and sheath liquid (p/n: H3301-1020) were provided by HMT, Japan.

Canavanine was identified by MassHunter WorkStation Qualitative Analysis B.05.00 (Agilent Technologies, USA). The raw data from CE/TOF-MS were re-analyzed by MassHunter WorkStation Quantitative Analysis B.06.00 software (Agilent Technologies, USA) to obtain stable data from samples. Data processing was conducted by extraction of the signal peak. The normalized of migration time was done by using internal standard. Comparison the value of m/z and normalized migration time of the internal standards were conducted for peaks alignment. Statistical significance was determined by ANOVA, followed by Tukey test at 5% probability.

3. Result and Discussion

Canavanine is non-protein amino acid (Rosenthal, 1982). Amino acid is known to be involved in plant response to pathogen infection (Dulermo et al., 2009; Rojas et al., 2014). Infection by *R. solani* induced the decrease of amino acid pool simultaneously

with the increase of amino acid non-protein in soybean sprout (Aliferis et al., 2014). Based on previous research, *R. solani* infection did not affect to the total amino acid of rice lines. However, as shown in Chapter 2, several amino acid metabolites showed change due to *R. solani* infection (Suharti et al., 2016). A similar case may occur to non-protein amino acid.

In this study, canavanine was detected in rice lines by using CE/TOF-MS in positive ion mode at migration time 8.687 min and mass to charge ratio at 177.0995 m/z. Since the charge of cation in the mass spectrometer is almost 1, the mass to charge ratio is equal to the molecular ion and molecular weight. Therefore, molecular ion and molecular weight of canavanine are 177.0995 m/z. These values are same with the results of canavanine determination in alfalfa by using NMR-ESI-MS (Keshavan et al., 2005) and legume by using LC/MS (Cai et al., 2009).

The two-way ANOVA showed that canavanine content affected by the rice line, inoculation treatment, and observation time (Table 4.1.). This evident indicates that canavanine may play a role to promote the resistance of 32R due to *R. solani* infection. Based on the time course of change (Fig. 4.1), both rice lines showed a different response in canavanine accumulation due to *R. solani* infection. Canavanine showed high in 32R than 29S. This phenomenon indicated that canavanine may involve in the defense mechanism of rice line as pathogen growth inhibitor. As reported by Samborski and Forsyth (1960), canavanine is recognized as a remarkable inhibitor in the development of rust disease on wheat. Moreover, a study of non-protein amino acid observed that canavanine able to protect plants from fungal pathogen infection (Simola and Lönnroth, 1979).

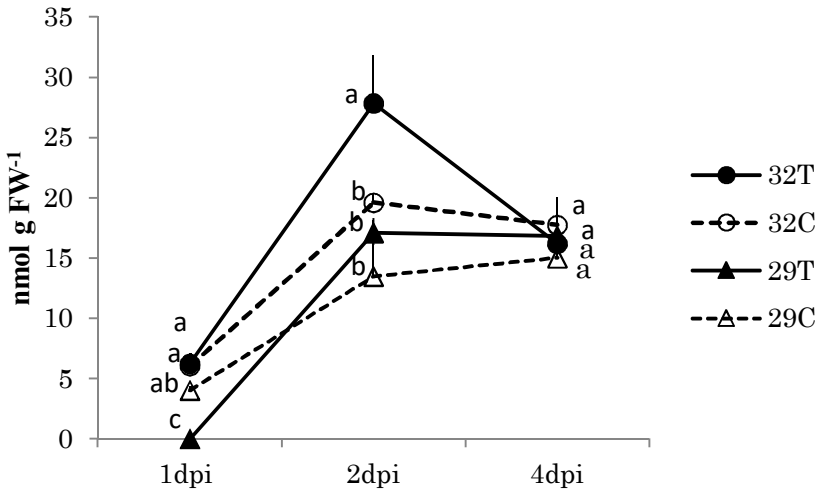

Fig. 4.1. Time course changes of canavanine (nmol g FW^{-1}) at 1dpi, 2dpi, 4dpi in 32R and 29S inoculated by *R solani* (●, ▲), and mock inoculated (○, △).

Table 4.1. Two way ANOVA and effect of interaction on the significant changes of canavanine

Parameter	Rice Line (R)	Inoculation Treatment (I)	Observation Time (O)	R*I	R*O	I*O	R*I*O
F	**20.531**	**6.161**	**60.095**	0.043	**7.615**	**5.383**	**4.421**
P-value	**<0.001**	**0.020**	**<0.001**	0.588	**0.020**	**0.011**	**0.045**

R*I is interaction between rice line and inoculation treatment, R*O is interaction between rice line and observation time, I*O is interaction between inoculation treatment and observation time, and R*I*O is interaction of three factor consist of rice line, inoculation treatment, and observation time. Bold numerics are statistical significance at $p < 0.05$.

The abundance of canavanine in 32R may associate with the function of canavanine as pathogen growth inhibitor. It is reported that canavanine has antimicrobial and antifungal activity to inhibit the growth of *Bacillus cereus* and *Peronospora tabacina* (Emmert et al., 1998; Sheperd and Mandryx, 1964). The abundance of canavanine in 32R also associated with plant resistance. Canavanine can act as antimetabolite to inhibit the arginine metabolism in the plant (Nakajima et al., 2001). The state of arginine in 32R and 29S were confirmed by Suharti et al (2016) in Chapter 2 which explained that the level of arginine in 32R showed lower than 29S. Arginine plays an important role as a storage form of organic nitrogen and their mobilization in the plant (Winter et al., 2015). High nitrogen content in the plant will enhance plant susceptibility to pathogen infection (Agrios, 1997). Thus, it suggested that canavanine has opposite effect with arginine in rice lines.

4. Summary

Based on this study, canavanine was successfully identified in rice leaves by using CE/TOF-MS. This study suggested the involvement of canavanine as plant resistance due to *R. solani* infection. By knowing the importance of canavanine in resistance mechanism, it is necessary to confirm the relationship between canavanine and *R. solani.* In the future, the study of canavanine effect on *R. solani* will be developed.

Chapter 5

General discussion

Plant pest and disease are perturbation in rice production. The frequent of rice cultivation and its distribution areas around the world generate the rice plant as the main hosts of various pests and diseases. According to Ou (1995), 64% of rice major disease is caused by fungal pathogens. One of rice plant disease is sheath blight diseases which caused by fungal pathogen, *R. solani.* The disease was first discovered in Japan in 1910 and economically important to increase the yield losses (Lee and Rush, 1983). Until now, no major resistance gene against *R. solani* has been reported. The resistance mechanism of rice to *R. solani* is determined by minor gene (polygene). In order to control rice sheath blight disease, two rice lines, 32R and 29S were developed by Wasano et al. (1985). These rice lines were presented as resistance and susceptible to sheath blight disease, respectively (Wasano and Hirota, 1986). 32R and 29S with different resistance characteristics are ideal to be used as the material to determine the various aspects of resistance mechanism in rice to sheath blight disease.

In previous research, both rice lines were used to observe metabolic and enzymatic changes as plant response due to *R. solani* infection (Danson, 1999; Mutuku and Nose, 2012). Resistant and susceptible plants have different response against pathogen infection. The plant resistance levels are depending on the resistance gene owned by the plant (Eckardt, 2002). Rice resistance to sheath blight disease is determined by polygene or minor gene which designated as quantitative trait loci (QTL). Gaihre and Nose (2011) was successfully to examine the QTL of resistance genes in

32R. Unfortunately, the QTL only determine the gene position on the genome and the quantitative effect on the resistance. The QTL is not directly correlated with the genes observed. Therefore, it is necessary to study the resistance mechanism more intensively. Through present study, resistance mechanisms of rice lines against *R. solani* infection were investigated comprehensively by metabolomic analysis approach.

The metabolomic is a part of omics studies aiming to detect the genes (genomics), mRNA (transcriptomics), protein (proteomics) and metabolite (metabolomic) in a biological sample (Horgan and Kenny, 2011). The omics principle relies on central dogma of biology which stated that the genetic information from DNA transcribes into mRNA. Subsequently, mRNA translates into protein that mostly have a function as enzyme involved in metabolic pathway (Sawada and Aoki, 2014). The disruption of the gene can cause the alteration in the transcript process. Furthermore, it will provide a change in enzyme levels, and ultimately will affect the metabolite profile. This explanation illustrates that omics studies are interrelated and complementary. Compared with other omics studies, metabolomic is the closest link to the phenotype. Therefore, the metabolomic study is necessary to be conducted to observe the rice response due to *R. solani* infection through phenotype trait approach.

Based on the study conducted in rice plants infected by *R. solani*, metabolomics provides three functions. The first function is metabolomic can be used as a tool to identify the primary and secondary metabolites. The second function is metabolomic can be used to describe and explain the metabolism occur in plant samples. The third function, metabolomic in this study can be used to observe the resistance mechanism of rice lines to *R. solani* infection.

As the first function, the metabolomic in this study is used as a tool to detect

primary and secondary metabolites of the samples by MassHunter software support. Hundreds of metabolites (Table 2.1 and 3.1) have been identified with the support of public metabolome database. The intensity of metabolites in each rice line was different as shown in hierarchical cluster (Figs. 2.2 and 3.2). Subsequently, the infection of *R. solani* provides the change of those metabolites. The metabolite changes may relate to the ability of plants to defend themselves against pathogen infection (Mazid et al., 2011; Rojas et al., 2014). Based on metabolomic study in positive ion mode (Chapter 2), the primary and secondary metabolites were distributed in branch of TCA-glycolysis pathway (Figs. 2.4 and 2.5). The primary metabolites generally includes in amino acids group. The total amino acids in two rice lines were not significantly different and not affected by the presence of *R. solani* infection. However, specific amino acids showed up-down regulated in both rice lines (Fig 2.3). The changes of specific amino acid metabolism may influence the plant due to *R. solani* infection. Meanwhile, the secondary metabolites observed as phenolic compound in 32R showed increased due to *R. solani* infection. In the metabolomic study by using negative ion mode (Chapter 3), the primary metabolites consist of nucleotides and organic compounds were observed in the samples. In addition, secondary metabolites generally grouped in benzenoid and phenylpropanoid (Figs. 3.3. and 3.4).

Although the metabolism of rice plant has been generally known, it may alter due to plant-pathogen interactions (Rojas et al., 2014). The change of metabolism in rice plant infected by pathogen has been acknowledged by metabolomic studies (Jones et al., 2010; Sana et al., 2010). By this study, the metabolism of rice lines infected by *R. solani* can be explained in more detail. Moreover, it is reported that metabolomic can increase the understanding of primary and secondary plant metabolism (Kusano et al., 2011;

Wang et al., 2015; Yang et al., 2014). Based on this study, amino acid metabolism, respiration, nitrogen assimilation, photorespiration, and lignifications showed different between each rice line. Subsequently, the infection of plant by *R. solani* causes the change of metabolism. As shown in Chapter 2, specific amino acid, such as GABA, glutamate, histidine, phenylalanine, serine, tryptophan, and tyrosine showed higher in 29S than 32R inoculated by *R. solani* (Fig. 2.7). The changes of specific amino acid metabolism may influence the plant due to *R. solani* infection. Furthermore, this phenomenon suggested the relationship between specific amino acid and susceptibility of rice plants to *R. solani*. In Chapter 3, the numbers of nucleotides, organic compounds and phenols were identified comprehensively in both rice lines. Nucleotides such as ADP and IMP showed up-down-regulated in 32R relative to 29S-*R. solani* inoculated (Fig. 3.5). Furthermore, it was revealed that each nucleotide associate with the changes of metabolism due to *R. solani* infection. The increase of ADP in 32R due to *R. solani* infection may associate with the increase of respiration. In this case, the respiration is dealing with plant response to provide the energy for plant defense against pathogen. The abundance of IMP in 29S inoculated by *R. solani* showed an increase of nitrogen assimilation which increases the disease severity. As shown in Chapter 3, glyceric acid was found abundance in 32R-mock inoculated. Probably, glyceric acid was characterized the resistance rice line. Glyceric acid is related with photorespiration. Subsequently, it will maintain the balance of ROS from cell death due to *R. solani* infection. The lignification also occurs and detected by metabolomic. The lignification is a metabolism of secondary metabolites as plant response to pathogen infection (Lattanzio et al., 2006). As shown in Figure 3.9, *R. solani* infection increased the intensity of phenols compound such as 2,5-dihydroxybenzoic acid, salicylic acid and

p-hydroxybenzoic acid in both rice lines. Phenol compound is important metabolites in lignin synthesis. However, 32 showed different response than 29S. In the 32R, plant was expressed the resistance due to *R. solani* infection. While in the 29S showed opposite occurrence. Increase of jasmonic acid probably related to the lignin accumulation due to *R. solani* infection. Jasmonic acid increment trend showed synergy with the lignin accumulation as research result conducted by Mutuku (2012). In 32R, the increasing of jasmonic acid leads to the lignin accumulation as plant defense mechanism.

Based on this study, metabolomic can determine the phenomenon in metabolism of rice against *R. solani* infection. Metabolites related to the mechanism were identified by comparing the resistant and susceptible rice lines and its response due to *R. solani* infection using volcano plot analysis. Based on metabolomic studies as shown in Chapter 2, 32R contained metabolites which provide resistance prior to the infection of *R. solani*. As shown in Fig. 2.3, chlorogenic acid was up regulated in 32R due to *R.solani* infection. Based on the time course change (Fig. 2.8), chlorogenic acid showed higher in 32R-mock treatment showed than 29S in the same treatment. Chlorogenic acid is known as secondary metabolites found in higher plants as resistance determinant. According to Bostock et al. (1999) and Wojciechowska et al (2014), chlorogenic acid in resistant plant is higher than susceptible. At the same time, *R. solani* infection triggered the increase of chlorogenic acid in 32R (Fig. 2.8). Thus, it may induce the resistance of 32R. In opposite, pipecolic acid was identified as amino acid with high p-value and fold change in 29S compare with 32R (Fig. 2.3). Pipecolic acid is known as important metabolite involved in plant resistance to pathogens (Vogel-Adghough et al., 2013). By knowing the abundance of pipecolic acid in 29S, the role of HR and SAR as plant defense mechanism in plant resistance to necrotropic

pathogen can be revealed. The result of Chapter 3 showed the role of mucic acid as important metabolite in resistance mechanism (Fig. 3.8). Mucic acid is related with the accumulation of pectin, a main substance in the formation of primary wall. It is known to play a role in plant immunity or known as basal resistance of plant to pathogen invasion (Doughari, 2015; Malinovsky et al., 2014).

The identification of non-target metabolite has been done by metabolomic as shown in Chapter 2 and 3. In order to identify the metabolite target, research shown in Chapter 4 has been conducted to determine the presence of canavanine in the leaves of rice. Canavanine is metabolite generally found in legume (Downum et al., 1983). The information about canavanine in the rice is very limited. Based on research result, massHunter Quantitative analysis software was successful to obtain stable data for canavanine. This type analysis is focused on the target metabolite. The research showed that canavanine affected by genotype, inoculation and observation time (Table 4.1). Furthermore, it suggested that canavanine involve in the interaction between rice line and *R. solani*, especially in the resistance mechanism of 32R to *R. solani* infection.

As a conclusion (Fig. 5.1), two rice lines (32R and 29S) used in this study showed different resistance mechanism as plant response against *R. solani*. Based on the research result, two rice lines generated two types of resistance mechanisms; 1) resistance mechanism of rice lines originated from the plant prior plant-pathogen interaction, 2) resistance mechanism induced by the pathogen infection. Prior to *R. solani* infection, 32R showed the increase of photorespiration and lignifications. After *R. solani* inoculation, 32R showed the increase of respiration, sugar accumulation, primary-secondary cell wall, antimicrobial activity and anti-metabolite to inhibit the nitrogen mobilization. In 29S, the resistance mechanism was not detected prior to *R.*

solani infection. Meanwhile, *R. solani* inoculation induced the activation of ROS activity, lignifications, photorespiration, SAR activity, and nitrogen assimilation-mobilization. Both rice lines showed the activation of photorespiration. However, the photorespiration differences between 32R and 29S may generated by the ROS mechanism. In 32R, metabolites associated with ROS have a function as ROS controller to maintain the ROS threshold from necrosis or cell death in plant tissue. While in 29S, ROS can enhance the plant susceptibility. This study showed that *R. solani* inoculation can induce the lignifications in both rice lines (Danson, 1999; Mutuku, 2012). In addition, *R. solani* also able to trigger the primary cell wall formation to prevents the penetration of *R. solani* mycelium into host tissue. The differences of lignifications in 32R and 29S were shown in resistance response each rice lines. In 32R, plant response showed obviously. In other side, the plant response was not expressed strongly in 29S. As stated previously, the lignifications were occurred in both rice lines. However, the lignin accumulation in 32R was higher than 29S as defense mechanism. Probably, the plant signaling influences the expression through lignin accumulation.

Previous research in our laboratory was successful to determine the QTL of sheath blight disease resistance in chromosome 7 related to cytokinin-*O*-glucosyltransferase (Gaihre et al., 2015). This finding has a relationship with metabolomic studies as shown in Fig 5.2. In the rice plant, cytokinin metabolism plays a role in nitrogen availability (Kamada-Nobusada et al., 2013). The research result suggested that the nitrogen accumulation in 32R lower than 29S. It is indicated by the low of IMP in 32R compared to 29S (Figure 3.7). In addition, several specific amino acids showed lower in 32R than 29S. Based on this finding, the QTL of sheath blight disease resistance encode the cytokinin-*O*-glucosyltransferase in 32R is expected to

inhibit the genes expression of amino acid and nitrogen accumulation. Further, it suggested leading the defense mechanism of 32R due to *R. solani* infection. QTL is linked to the gene related to the phenotype trait of the plant. Simultaneously, the metabolomic is more appropriate for research on phenotype compares with other omics studies. Both studies are mutually supportive and complementary in order to observe the phenotype trait of the plant infected by *R. solani* and attributed to the polygene effect.

According to Rochfort (2005), metabolite is the result of interaction between the genome and the environment. Through the study, the correlation between metabolite, plant resistance, and pathogen infection in the case of rice lines infected by *R. solani* have been revealed. The different response between resistant and susceptible rice line are more distinguishable. Metabolite changes occur on plants infected with *R. solani* affects the metabolism involving as the process of plant defense mechanism. The genotypes of plants and the interactions between plants and pathogens will determine metabolite profiles and the regulation of metabolism. The number of metabolite changes after the *R. solani* infections indicate that there might be many genes responsible for resistance of rice plants infected with *R. solani*. This information is necessary to understanding the plant physiology related to the polygene resistance. Nevertheless, this finding must be confirmed further by gene expression analysis by transcriptomic approach using micro array.

Finally, the metabolomic study is able to provide knowledges about the resistance and susceptibility of rice lines by using limited method. This information is very useful to explain the mechanism of resistance in resistant and susceptible rice lines. Furthermore, it can be used as a reference in the engineering of rice resistant variety to *R. solani*.

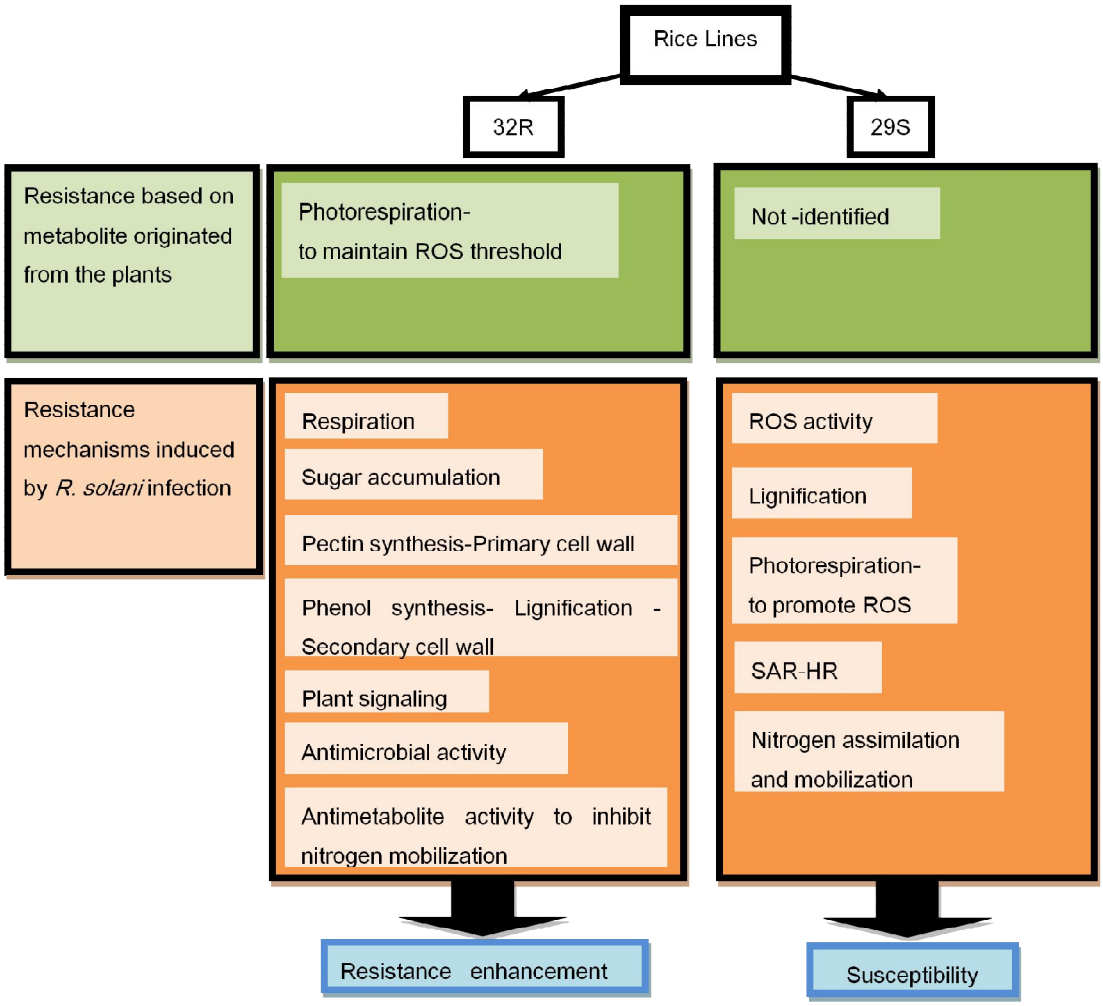

Fig. 5.1. Schematic presentation of the metabolomic study conclusion.

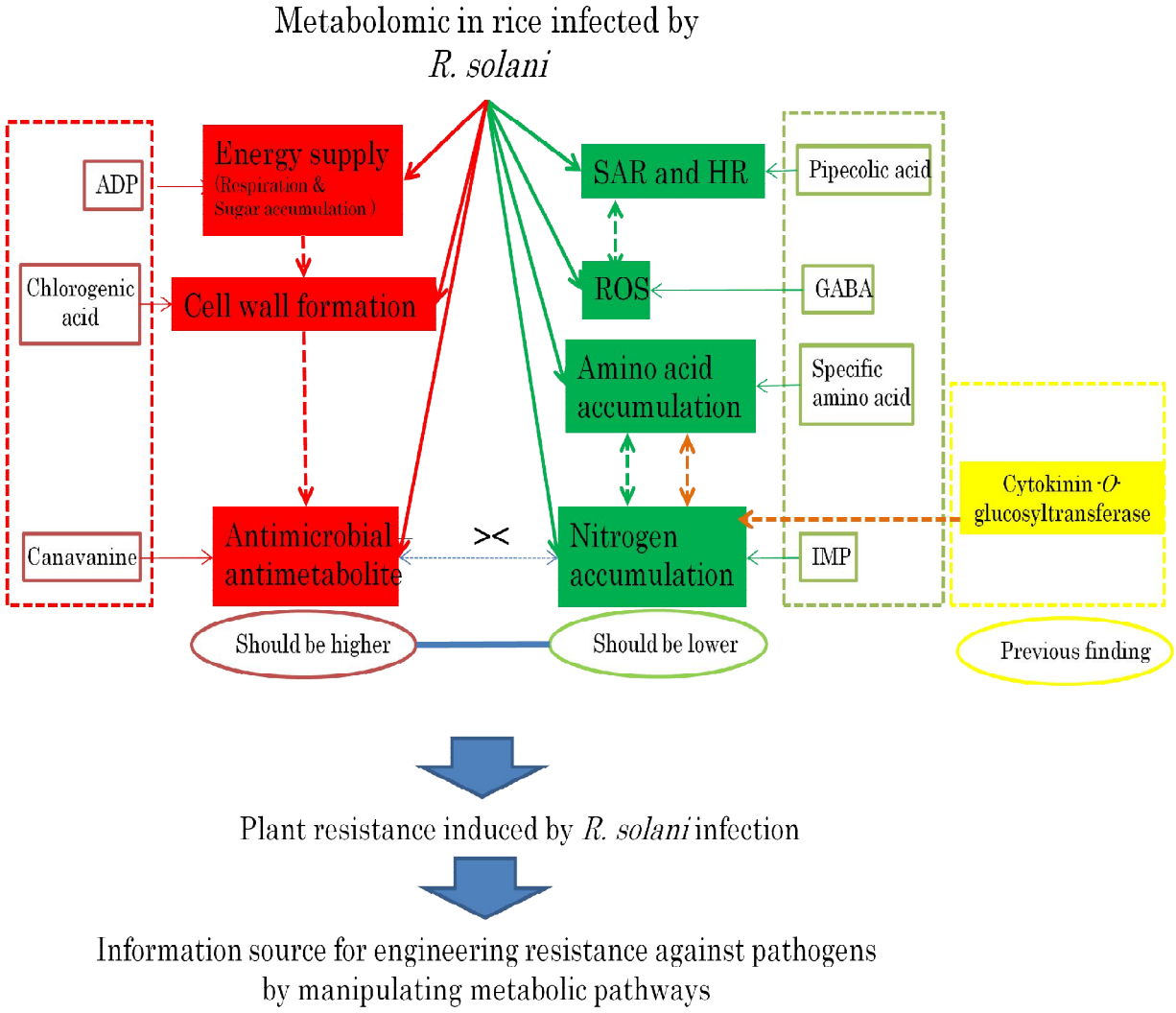

Fig. 5.2. Schematic of metabolomic study on rice lines infected by *R. solani* and its relationship with QTL encode cytokinin-*O*-glucosyltransferase.

Future studies

Sheath blight disease caused by *R. solani* is an important disease in the rice plant. The various plant management has been attempted to be introduced, including resistant varieties. Nevertheless, the polygenic resistance of rice plant to *R. solani* caused many resistance genes involved and become the constraint in genetic engineering of plant resistant.

The metabolomics study has been successful to reveal the 32R and 29S response against *R. solani* as presented in Chapter 2, 3, and 4. The increase of several metabolites explained the metabolic changes as plant defense of rice line due to *R. solani* infection. This study showed the differences of two rice lines characteristic. The observation time on this study was conducted at 1, 2, and 4dpi. It is suggested that the metabolic changes may occur before 1dpi. Therefore, metabolomics studies in plants with observation time of less than 1dpi should be done in the future (Fig. 5.3).

Metabolomics study was able to reveal the up-down-regulated metabolite in rice lines infected by *R. solani* (Chapter 2 and 3). Nevertheless, the expression of plant resistance genes can not be appeared caused by stop or broken. Based on this phenomenon, the study of gene expression by using real-time quantitative PCR (qPCR) or microarray is very important. The study of gene expression or known as transcriptomic is a part of omics that suitable to observe genomic in mRNA level (Fig. 5.3). The result will complete the phenotype trait study from QTL analysis and metabolomic. This future study is appropriate with the central dogma of biology and promotes the metabolomic studies to provide the comprehensive information on plant resistance.

In the Chapter 4, canavanine was detected in rice plants. This finding may explain the increased of antimicrobial activity which induced by *R. solani*. Canavanine study in rice plant and the interaction with fungal pathogen is still limited. Based on this, it is necessary to conduct the research on the interaction between canavanine and *R. solani*, especially in the canavanine ability to inhibit the growth and development of *R. solani* (Fig. 5.3).

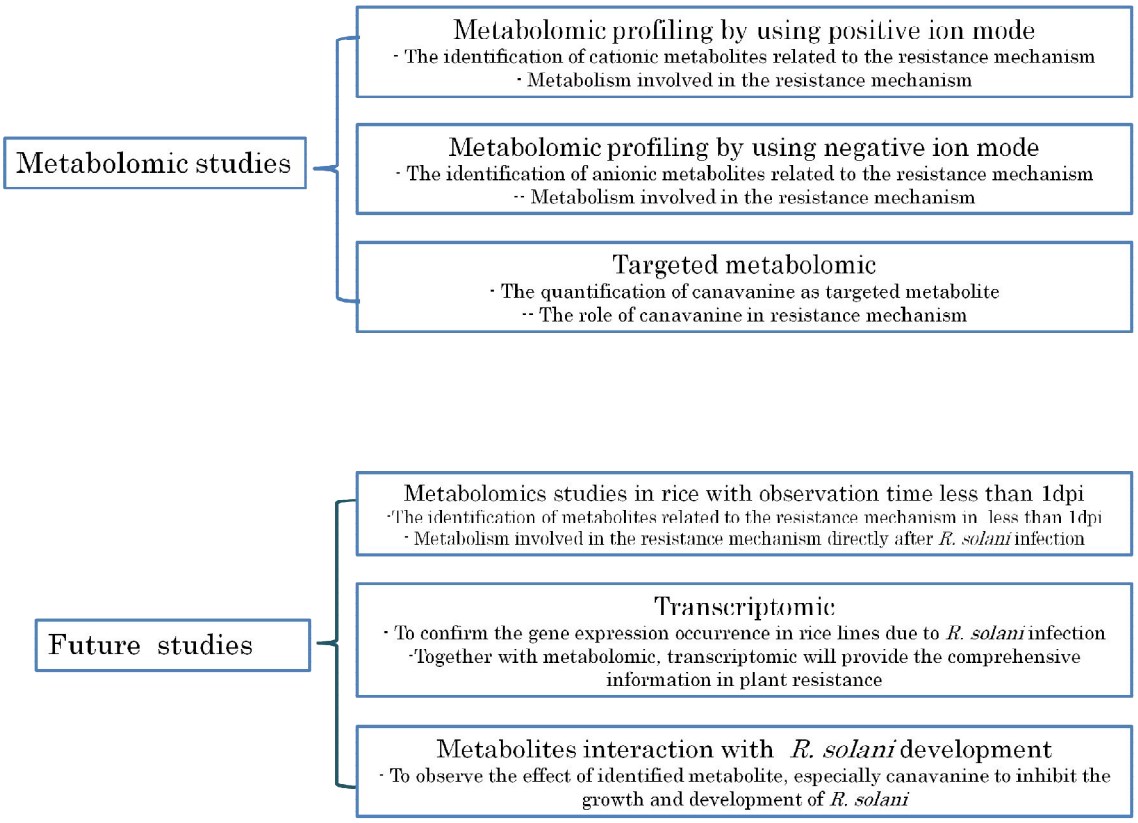

Fig. 5.3. Schematic of metabolomic studies and future studies.

Summary

Rice is staple food for the most of the world's population. In rice cultivation, sheath blight disease caused by *Rhizoctonia solani* is economical important. Cultural control with resistant varieties is one of techniques to control the disease. However, the engineering on resistant varieties of rice have problems due to its resistance to *R. solani* determined by many genes (polygene). In this regards, two rice lines have developed in our laboratory, 32R is resistant to *R. solani* and 29S is susceptible, respectively. The objective of this study is to reveal the resistance mechanism as response of two rice lines due to *R. solani* infection by using metabolomic. This study objective is to explain the rice lines response to *R. solani* on the view points of metabolites involved and metabolic regulation as resistance mechanism, as well as the role of metabolomic in resistance mechanism.

Analysis on plant resistance by metabolomic approach is based on the ion mode, positive and negative ion mode. Metabolomic study using positive ion mode has successfully revealed the increase of chlorogenic acid in 32R due to *R. solani* infection that leads to the formation of lignifications and secondary cell walls. Although total amino acid content are similar in infected 32R and 29S, specific amino acid such as GABA, glutamate, glycine, histidine, phenylalanine, serine, tryptophan, and tyrosine accumulated in 29S due to *R. solani* infection. This occurence suggests the relationship between specific amino acid and susceptibility of rice plants to *R. solani*. In addition, the amino acid accumulation in 29S suggests providing a good condition for inner cell as increasing a nutrient source for fungal growth. The pipecolic acid showed higher in 29S than 32R due to *R. solani* infection. It may support the hypersensitive response

(HR) which leads to the susceptibility in plant to the infection of necrotrophic fungal pathogen. The metabolomic study using positive ion mode also revealed the existence of canavanine as potential antimetabolite and antimicrobial in 32R due to *R. solani* infection.

In the metabolomics study using negative ion mode, the high of glyceric acid in 32R indicates that photorespiration occurs to maintain the ROS threshold. The *R. solani* infection to 32R increases the carbohydrate metabolites which alter the sugar accumulation. Additionally, the infection of *R. solani* in 32R increases the ADP that suggested the increment of respiration. In 32R infected by *R. solani,* the mucic acid increased that leads to the activation of pectin synthesis in primary cell wall formation. Jasmonic acid activation in 32R after infected by *R. solani* suggested to the plant signaling activation that leads to lignifications and secondary cell wall formation. In the 29S, the infection of *R. solani* causes to the increase of inosine monophosphate (IMP) relating to nitrogen assimilation and mobilization.

The study shows that each rice line has different responses as resistance mechanism against *R. solani* infection. Metabolomic study is able to complement the previous studies on plant resistance to *R. solani*. Finally, metabolomic study can provide a knowledge and information in order to explain the mechanism of resistance in resistant and susceptible rice lines by using simple and fast method. Furthermore, this information is expected to be used as a reference in the engineering of rice resistant variety to *R. solani.*

Abstract in Japanese

世界の主要作物のひとつである水稲の栽培において、*Rhizoctonia solani* によってもたらされる紋枯病は地球温暖化に伴って拡大する主要病害のひとつで、抵抗性品種の開発は喫緊の課題となっている。特に、イネ紋枯病抵抗性に対する主導遺伝子は存在せず、ポリジーン抵抗性のみが知られ、抵抗性品種の開発にはポリジーン由来の複雑な抵抗性メカニズムの解明が求められている。本研究では、和佐野らによって開発されたポリジーン由来の抵抗性系統 32R と感受性系統 29S について、CEMS を用いたメタボローム解析を実施し、抵抗性・感受性の代謝メカニズムについて明らかにした。

CEMS のポジティブイオンモードを用いた解析において、抵抗性系統 32R においては *R. solani* 感染にともない細胞壁におけるリグニン蓄積や二次細胞壁の形成をもたらすクロロゲン酸が増大した。一方、感受性系統 29S においては、総可溶性アミノ酸含量は、32R と同様であったにも関わらず、*R. solani* の栄養源となる GABA、グルタミン、ヒスチジン、チロシン等の特異的なアミノ酸が増大し、菌の生長を促す細胞内環境が形成されることが明らかになった。また、感染後の 29S においては、植物において壊死性菌に対する過敏反応（HR）を誘導するピペコリン酸の増大が観察された。特に、ナタマメで発見された昆虫毒のカナバミンが 32R で感染後に生成されることが明らかになった。

ネガティブイオンモードでの CEMS 分析においては、光呼吸の増大を反映したグリセンリン酸の増大が感染後の 32R で観察され、感染にともなう活性酸素

の増大が抑制されることが示唆された。また、32R においては、感染にともない呼吸の増大を示すアデノシン二リン酸（ADP）の増大や菌糸の細胞への侵入を阻む細胞壁の形成を促すムチン酸の増大が観察された。さらに、感染後の 32R におけるジャスモン酸の増大は、細胞壁におけるリングニン蓄積や二次細胞壁形成を促すシグナル伝達機構の誘導を示すものである。他方、感受性系統 29S においては、窒素同化を促進するイノシン一リン酸（IMP）が増大することが明らかになった。

以上 CEMS を用いたメタボローム解によって、抵抗性系統 32R と感受性系統 29S は、*R. solani* 感染に対し、今迄に知られていた細胞壁のリグニン蓄積に関連した代謝を含め、広範囲にわたり、夫々に特異的な代謝反応を示すことが明らかになった。また、本研究の成果は、今までに明らかにされていない紋枯病抵抗性及び感受性遺伝子を示唆し、今後の代謝工学的手法を用いての抵抗性品種の開発に貢献するものと考えられる。

References

Agrios, G.N. 1988. Plant Pathology, third ed. Academic Press, Inc., New York. 803 pp.

Agarwal, D.K. 2010. *Rhizoctonia* D. C: Taxonomy, Ecology and Management. In. Mukerji, K.G., Manoharachary (Eds.), Taxonomy and Indian Fungi. International Publishing House, New Delhi, pp. 19-49.

Ahmad, S. 2013. Plant metabolomics: techniques, applications, trends, and challenges. In. Barh, D., Zambare, V., Azevedo, V. (Eds.), Omics: applications in biomedical, agricultural, and environmental sciences. CRC Press, Boca Raton, pp. 451-480.

Akhtar, J., Jha, V.K., and Lal, H.C. 2011. Post-infectional phenolic changes in maize due to *Rhizoctonia solani* f. sp. sasakii causing banded leaf and sheath blight. *Indian Phytopathology* 64: 261-264.

Ali, H.Z., and Nadarajah, K. 2013. Evaluating the efficacy of *Trichoderma* isolates and *Bacillus subtilis* as biological control agents against *Rhizoctonia solani. Research Journal of Applied Sciences* 8:72-81.

Aliferis, K.A., Faubert, D., and Jabaji, S. 2014. A metabolic profiling strategy for the dissection of plant defense against fungal pathogens. *PLoS ONE* 9: e111930.1-9

Aliferis, K.A., and Jabaji, S. 2012. FT-ICR/MS and GC-EI/MS metabolomics networking unravels global potato sprout's responses to *Rhizoctonia solani* infection. *PLoS ONE.* 7: e42576.1-13.

Ansari, M.A., Anurag, A., Fatima, Z., and Hameed, S. 2013. Natural phenolic compounds: a potential antifungal agent. In. Mendez-Vilas, A. (Ed.). Microbial pathogens and strategies for combating them: science, technology, and education, vol. 2. Formatex Research Center, Spain, pp. 1189–1195.

Archibald, R.M. 1946. Colorimetric determination of canavanine. *Journal of Biological Chemistry* 165: 169-178.

Barna, B., Fodor, J., Harrach, B.D., Pogány, M., and Király Z. 2012. The Janus face of reactive oxygen species in resistance and susceptibility of plants to necrotrophic and biotrophic pathogens. *Plant physiology and biochemistry* 59: 37-43.

Barnwal, M.K., Kotasthane, A., Magculia, N., Mukherje, P.K., Savry, S., Sharma, A.K., Singh, H.B., Singh, U.S., Sparks, A.H., Variar, M., and Zaidi, N. 2013. A review on crop losses, epidemiology and disease management on rice brown spot to

identify research priorities and knowledge gaps. *European Journal Plant Pathology* 136: 443-457.

Bartsch, M., Bednarek, P., Vivancos, P.D., Schneider, B., Roepenack-Lahaye, E.V., Foyer, C.H., Kombrink, E., Scheel, D., and Parker, J.E., 2010. Accumulation of isochorismate-derived 2,3-dihydroxybenzoic 3-*O*-*β*-D-xyloside in *Arabidopsis* resistance to pathogens and ageing of leaves. *Journal of Biological Chemistry* 285: 25654–25665.

Berg, J.M., Tymoczko, J.L., and Stryer, L., 2002. Biochemistry, fifth ed. W.H. Freeman, New York. 974 pp.

Bhuiyan, N.H., Selvaraj, G., Wei, Y., and King, J. 2009. Role of lignification in plant defence. *Plant Signaling Behavior* 4: 158-159.

Bogan, K.L., and Brenner, C. 2010. 5'-Nucleotides and their new roles in NAD^+ and phosphate metabolism. *New Journal of Chemistry* 34: 845-853.

Bolton, M.D., 2009. Primary metabolism and plant defense—fuel for the fire. *Molecular Plant-Microbe Interactions* 22: 487–497.

Bolwell, G.P., and Daudi, A. 2009. Reactive oxygen species in plant–pathogen interactions. In. Del Río L. A. and Puppo A. (Eds.), Reactive oxygen species in plant signaling. Springer-Verlag, Heidelberg. pp. 113-133.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72: 248-254.

Bostock, R.M., Wilcox, S., Wang, G., and Adaskaveg, J.E. 1999. Suppression of *Monilia fructicola* cutinase production by peah fruit surface phenolic acids. *Physiological and Molecular Plant Pathology* 54: 37-50.

Bubna, G.A., Lima, R.B., Zanardo, D.Y.L., dos Santos, W.D., Ferrarese, M.L.L., and Ferrarese-Filho, O. 2011. Exogenous caffeic acid inhibits the growth and enhances the lignification of the roots of soybean (*Glycine max*). *Journal of Plant Physiology* 168: 1627– 1633.

Cai, T., Cai, W., Zhang, J., Zheng, H., Tsou, A.M., Xiao, L., Zhong, Z., and Zhu, J. 2009. Host legume-exuded antimetabolites optimize the symbiotic rhizosphere. *Molecular Microbiology* 73: 507-517.

Cheynier, V. 2012. Phenolic compounds: from plants to foods. *Phytochemistry Reviews* 11: 153-177.

Conrath, U. 2006. Systemic acquired resistance. *Plant Signaling Behavior* 1: 179-184.

Datta, K., Velazhahan, R., Oliva, N., Ona, I., Mew, T., Khush, G.S., Muthukrishnan, S., and Datta, S.K. 1999. Over-expression of the cloned rice thaumatin-like protein (PR-5) gene in transgenic rice plants enhances environmental friendly resistance to *Rhizoctonia solani* causing sheath blight disease. *Theoretical and Applied Genetics* 98: 1138-1145.

Danson, J. 1999. Studies on carbohydrate and secondary metabolism of rice plants (*Oryza sativa*) in relation to resistance responses to sheath blight fungus (*Rhizoctonia solani*) infection. PhD Thesis, Kagoshima University, Japan. 130 pp.

Danson, J., Wasano, K., and Nose, A. 2000. Infection of rice plants with the sheath blight fungus causes an activation of pentose phosphate and glycolytic pathways. *European Journal of Plant Pathology* 106: 555-561.

Dempsey, D.A., Vlot, A.C., Wildermuth M.C., and Klessig, D.F. 2011. Salicylic acid biosynthesis and metabolism. *The Arabidopsis Book* 9:e0156.1-24.

Dettmer, K., Aronov, P.A., and Hammock, B.D. 2007. Mass spectrometry-based metabolomics. *Mass Spectrometry Reviews* 26: 51–78.

Dixon, R.A., Achnine, L., Kota, P., Liu, C.J., Reddy, M.S.S., and Wang, L. 2002. The phenylpropanoid pathway and plant defence-A genomics perspective. *Molecular Plant Pathology* 3: 371–390.

Dong, X., Wang, M., Ling, N., Shen, Q., and Guo, S. 2016. Potential role of photosynthesis-related factors in banana metabolism and defense against *Fusarium oxysporum* f. sp. *cubense*. *Environmental and Experimental Botany* 129: 4-12.

Douglas, C.J. 1996. Phenylpropanoid metabolism and lignin biosynthesis: from weeds to trees. *Trends Plant Science* 1: 171-178.

Doughari, J.H. 2015. An overview of plant immunity. *Journal of Plant Pathology & Microbiology* 6: 1-11.

Downum, K.R., Rosenthal, G.A., and Cohen, W.S. 1983. L-arginine and L-canavanine metabolism in jack bean, *Canavalia ensiformis* (L.) DC. and soybean, *Glycine max* (L.) Merr. *Plant Physiology* 73: 965-968.

Drame, K.N., Manneh, B., and Ismail, A.M. 2013. Rice genetic improvement for abiotic stress tolerance in Africa. In. Wopereis, M. C. S., Johnson, D. E., Ahmadi, N., Tollens, E., and Jalloh, A. (Eds.). Realizing Africa's rice promise. Africa Rice Center (AfricaRice), Cotonou, Benin, pp. 144-160.

Dulermo, T., Bligny, R., Gout, E., and Cotton, P. 2009. Amino acid changes during sunflower infection by necrotrophic fungus *B. cinerea*. *Plant Signaling Behavior* 4: 859-861

Eckardt, N.A. 2002. Plant disease susceptibility genes? *The Plant Cell* 14: 1983-1986

Ekeleme, F., Kamara, A.Y., Oikeh, O., Chikoye, D., and Omoigut, L.O. 2007. Effect of weed competition on upland rice production in north-estern Nigeria. *African Crop Science Conference Proceedings* 8: 61-65.

Emmert E.A.B., Milner J.L., Lee J.C., Pulvermacher K.L., Olivares H.A., Clardy J., and Handelsman J. 1998. Canavanine from Alfalfa seeds on the population biology of *Bacillus cereus. Applied and Environmental Microbiology* 64: 4683-4688.

Engelsdorf, T., Horst, R.J., Pröls, R., Pröschel, M., Dietz, F., Hückelhoven, R., and Voll, L.M. 2013. Reduced carbohydrate availability enhances the susceptibility of arabidopsis toward *Colletotrichum higginsianum. Plant Physiology* 162; 225–238.

Fernie, A.R., Carrari, F., and Sweetlove, L.J. 2004. Respiratory metabolism: glycolysis, the TCA cycle and mitochondrial electron transport. *Current Opinion Plant Biology* 7: 254-261.

Fiehn, O. 2002. Metabolomics--the link between genotypes and phenotypes. *Plant Molecular Biology* 48:155-171.

Forde, B.G., and Lea, P.J. 2007. Glutamate in plants: metabolism, regulation, and signaling. *Journal of Experimental Botany* 58: 2339–2358.

Frazier, R.A., Nursten, H.E., and Ames, J.M., 2000. Capillary electrophoresis for food analysis: method development, Royal Society of Chemistry, Cambridge. 135 pp.

Gaihre, Y.R., and Nose, A. 2011. QTL analysis of sheath blight disease resistance in the rice line 32R derived from Tetep. *Japanese Journal of Crop Science* 80: 172-173.

Gaihre, Y.R., and Nose, A. 2013. High yielding capabilities and genetic variation in crossing of sheath blight disease resistant rice line. *Field Crops Research* 149: 133-140.

Gaihre, Y.R., Yamagata, Y., Yoshimura, A., and Nose, A. 2015. Identification of QTLs involved in resistance to sheath blight disease in rice line 32R derived from Tetep. *Tropical Agriculture and Development* 59: 54-160.

Green, N.E., Hadwiger, L.A. and Graham, S.O. 1975. Phenylalanine ammonia-lyase, tyrosine ammonia-lyase, and lignin in wheat inoculated with *Erysiphe graminis* f.sp. tritici. *Phytopathology* 65: 1071-1074.

Govrin, E.M. and Levine, A. 2000. The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. *Current Biology* 10: 751–757.

Hall, R., Beale, M., Fiehn, O., Hardy, N., Sumner, L., and Bino, R. 2002. Plant Metabolomics. *Plant Cell* 14: 1437-1440.

Hecker, R.J., Ruppel, E.G., Maag, G.W., and Rasmuson, D.M. 1975. Amino acids associated with Cercospora leaf spot resistance in sugarbeet. *Phytopathologische Zeitschrift* 82: 175-181.

Horgan, R.P., and Kenny, L.C. 2011. SAC review 'omic' technologies: genomics, transcriptomics, proteomics and metabolomics. *The obstetrician & Gynaecologist* 13: 189-195.

Hossain, M.K., Tze, O.S., Nadarajah, K., Jena, K., Bhuiyan, M.A.R., and Ratnam, W. 2014 Identification and validation of sheath blight resistance in rice (Oryza sativa L.) cultivars against *Rhizoctonia solani. Canadian Journal of Plant Pathology* 36: 482-490.

Igarashi, D., Izumi, Y., Dokiya, Y., Totsuka, K., Fukusaki, E., and Ohsumi, C. 2008. Reproductive organs regulate leaf nitrogen metabolism mediated by cytokinin signal. *Planta* 229: 633-644.

Jia, Y., Correa-Victoria, F., McClung, A., Zhu, L., Liu, G., Wamishe, Y., Xie, J., Marchetti, M. A., Pinson, S.R.M., Rutger, J.N., and Correll, J.C. 2006. Rapid determination of rice cultivar responses to the sheath blight pathogen *Rhizoctonia solani* using a micro-chamber screening method. *Plant Disease* 91: 485-489.

Jimenez-Garcia, S.N., Vazquez-Cruz, M.A., Guevara-Gonzalez, R.G., Torres-Pacheco, I., Cruz-Hernandez, A., and Feregrino-Perez, A.A. 2013. Current Approaches for Enhanced Expression of Secondary Metabolites as Bioactive Compounds in Plants for Agronomic and Human Health Purposes – a Review. *Polish Journal of*

Food and Nutrition Sciences 63: 67-78.

Johnson, R. 1981. Durable resistance: Definition of, genetic control, and attainment in plant breeding. *Phytopathology* 71: 567-568.

Jones, O.A.H., Maguire, M.L., Griffin, J.L., Jung, Y-H., Shibato, J., Rakwal., R., Agrawal, G.K., and Jwa, N-S. 2011. Using metabolic profiling to assess plant-pathogen interactions: an example using rice (*Oryza sativa)* and the blast pathogen *Magnaporthe grisea*. *European Journal Plant Pathology* 129: 539-554.

Kagale, S., Marimuthu, T., Kagale, J., Thayumanavan, B., and Samiyappan, R. 2011. Induction of systemic resistance in rice by leaf extracts of *Zizyphus jujuba* and *Ipomoea carnea* against *Rhizoctonia solani. Plant Signaling Behavior* 6: 919-923.

Kamada-Nobusada, T., Makita, N., Kojima, M., and Sakakibara, H. 2013. Nitrogen-Dependent Regulation of De Novo Cytokinin Biosynthesis in Rice: The Role of Glutamine Metabolism as an Additional Signal. *Plant and Cell Physiology* 54: 1881-1893.

Kangasjärvi, S., Neukermans, J., Li, S., Aro E.M., and Noctor, G. 2012. Photosynthesis, photorespiration, and light signalling in defence responses. *Journal of Experimental Botany* 63: 1619-1636.

Kappock, T.J., Ealick, S.E., and Stubbe, J.A. 2000. Modular evolution of the purine biosynthetic pathway. *Current Opinion in Chemical Biology* 4: 567-572.

Keijer, J. 1996. The initial steps of the infection process in *Rhizoctonia solani*. In. Sneh, B., Jabaji-Hare, S., Neate, S., and Dijst, G. (Eds.), Rhizoctonia species: taxonomy, molecular biology, ecology, pathology and disease control. Kluwer Academic Publishers, The Netherlands, pp. 149-162.

Keshavan, N.D., Chowdhary, P.K., Haines, D.C., and González, J.E. 2005. L-Canavanine made by *Medicago sativa* with quorum sensing in *Sinorhizobium meliloti*. *Journal of Bacteriology* 187: 8427-8436.

Kiet, H.V., and Nose, A. 2015. Effects of temperature on growth and photosynthesis in the seedling stage of the sheath blight-resistant rice genotype 32R. *Plant Production Science* 19: 246-256.

Kluger, B., Bueschl, C., Neumann, N., Stückler, R., Doppler, M., Chassy, A.W., Waterhouse, A. L., Rechthaler, J., Kampleitner, N., Thallinger, G.G., Adam, G., Krska, R., and Schuhmacher, R. 2014. Untargeted profiling of tracer-derived

metabolites using stable isotopic labeling and fast polarity-switching LC−ESI-HRMS. *Analytical Chemistry* 86: 11533−11537.

Kolch, W., Neusüss, C., Pelzing, M., and Mischak, H. 2005. Capillar electrophoresis-mass spectrometry as a powerful tool in clinical diagnosis and biomarker discovery. *Mass Spectrometry Reviews* 24: 959-977.

Krasuska, U., Andrzejczak O., Staszek P., Bogatek R., and Gniazdowska A. 2016. Canavanine Alters ROS/RNS Level and Leads to Post-translational Modification of Proteins in Roots of Tomato Seedlings. *Frontiers in Plant Science* 7: 1-15.

Kusano M., Fukushima A., Redestig H., and Saito K. 2011. Metabolomic approaches toward understanding nitrogen metabolism in plants. *Journal of Experimental Botany* 62: 1439-1453

Kushalappa, A.C., and Gunnaiah, R. 2013. Metabolo-proteomics to discover plant biotic stress resistance genes. *Trends Plant Science* 18: 522-531.

Lattanzio, V., Lattanzio, V.M.T., and Cardinali, A. 2006. Role of phenolic compounds in the resistance mechanisms of plants against fungal pathogens and insects. In. Imperato, F. (Ed.). Phytochemistry: Advances in Research. Research Signpost, Kerala, India, pp. 23-67.

Lawlor, D.W. 2002. Carbon and nitrogen assimilation in relation to yield: mechanisms are the key to understanding production systems. *Journal of Experimental Botany* 53, 773–787.

Lee, F.N., and Rush, M.C. 1983. Rice sheath blight: a major rice disease. *Plant Disease* 67: 829-832.

Lemire, J., Milandu, Y., Auger, C., Bignucolo, A., Appanna V.P., and Appanna V.D. 2010. Histidine is a source of the antioxidant, α-ketoglutarate, in *Pseudomonas fluorescens* challenged by oxidative stress. *FEMS Microbiology Letters* 309: 170-177.

Liang, Q., Wang, C., Li, B., and Zhang, A. 2015. Metabolic fingerprinting to understand theurapetic effects and mechanism of silybin on acute liver damage in rat. *Pharmacognosy Magazine* 11: 586-593.

Li, Z., Pinson, S.R., Marchetti, M.A., Stansel, J.W., and Park, W.D. 1995. Characterization of quantitative trait loci (QTLs) in cultivated rice contributing to field resistance to sheath blight (*Rhizoctonia solani*). *Theoretical and Applied*

Genetics 91: 382-388.

Lindhout, P. 2002. The perspectives of polygenic resistance in breeding for durable disease resistance. *Euphytica* 124: 217-226.

Marchetti M.A. 1983. Potential impact of sheath blight on yield and milling quality of short statured rice lines in the southern United States. *Plant Disease* 67:1 62-165.

Malinovsky, F.G., Fangel, J.U., and Willats, W.G.T. 2014. The role of the cell wall in plant immunity. *Frontiers in Plant Science* 5: 1-12.

Mazid, M., Khan, T.A., and Mohammad, F. 2011. Role of secondary metabolites in defense mechanism of plants. *Biology and Medicine* 3: 232-249.

Mengiste, T. 2012. Plant immunity to necrotrophs. *Annual Review of Phytopathology* 50: 267–294.

Millerd, A., and Scott, K.J. 1962. Respiration of the diseased plant. *Annual Review of Phytopathology* 13: 559-574.

Morkunas, I., Formela, M., Marczak, L., Stobiecki, M., and Bednarski, W. 2013. The mobilization of defence mechanisms in the early stages of pea seed germination against *Ascochyta pisi*. *Protoplasma* 250: 63–75.

Morkunas, I., and Ratajczak, L. 2014. The role of sugar signaling in plant defense responses against fungal pathogens. *Acta Physiology Plantarum* 36: 1607–1619.

Muthayya, S., Sugimoto, J.D., Montgomery, S., and Maberly, G.F. 2014. An overview of global rice production, supply, trade, and consumption. *Annals of the New York Academy of Science.* 1324: 7-14.

Mutuku, J.M. 2012. Molecular biology studies on resistance and susceptibility of rice to sheath blight disease caused by *Rhizoctonia solani* Kuhn. PhD Thesis, Kagoshima University, Japan. 165 pp.

Mutuku, J.M., and Nose, A. 2010. *Rhizoctonia solani* infection in two rice lines increases mRNA expression of metabolic enzyme genes in glycolytic, oxidative pentose phosphate pathways ans secondary metabolites. *Tropical Agriculture and Development* 54: 119-131.

Mutuku, J.M., and Nose, A. 2012. Changes in the contents of metabolites and enzyme activities in rice plants responding to *Rhizoctonia solani* Kuhn infection:

activation of glycolysis and connection to phenylpropanoid pathway. *Plant and Cell Physiology* 53: 1017-1032.

Naemi, S., Okhovvat, S.M., Javan-Nikkhah, M., Vágvölgyi, C., Khosravi, V., and Kredic, L. 2010. Biological control of *Rhizoctonia solani* AG1-1A, the causal agent of rice sheath blight with *Trichoderma* strains. *Phytopathologia Mediterrania* 49: 287−300.

Nakajima N., Hiradate S., and Fujii Y. 2001. Plant growth inhibitory activity of L-canavanine and its mode of action. *Journal of Chemical Ecology* 27:19-31.

Nagarajkumar, M., Bhaskaran, R., and Velezhahan, R. 2004. Involvement of secondary metabolites and extracellular lytic enzymes produced by *Pseudomonas fluorescens* in inhibition of *Rhizoctonia solani*, the rice sheath blight pathogen. *Microbiological Reserch* 159: 73-81.

Navajothy, A.F., Narayanaswamy, R., Ponniah, D., and Irudayaraj, V. 2011. High performance liquid chromatography for the detection and estimation of amino acids in relation to Fusarium wilt (panama disease) in banana. *International Multidisciplinary Research Journal* 1: 49-56.

Oropeza C., Alpizar L., Loyola-Vargas V.M., Quiroz J., and Scorer K.N. 1988. Determination of L-canavanine and L-canaline in plant tissues by high-performance liquid chromatography. *Journal of Chromatography* 456: 405-409.

Ou S.H., 1985. Rice Diseases. Second ed. Commonwealth Mycological Institute, Kew Surrey, UK. 380 pp.

Palloix, A., Ayme, V., and Moury, B. 2009. Durability of plant major resistance genes to pathogens depends on the genetic background, experimental evidence and consequences for breeding strategies. *New Phytologist* 183: 190–199.

Papademetriou, M.K. 2000. Rice production in the Asia-Pacific region: Issues and perspectives. In Papademetriou, M.K., Dent, F.J., Herath, E.M. (Eds.), Bridging the rice yield gap in the Asia-Pacific Region. Rap Publication: 2000/16. Food and the Agriculture Organization of the United Nations Regional Office for Asia and the Pacific, Bangkok, pp. 4-25.

Park, D.-S., Sayler, R.J., Hong, Y.-G., Nam, M.-H., and Yang, Y. 2008. A method for inoculation and evaluation of rice sheath blight disease. *Plant Disease* 92: 25-29.

Parlevliet, J.E., 2002. Durability of resistance against fungal, bacterial and viral pathogens; present situation. *Euphytica* 124: 147–156.

Petersen, M., Strack, D., and Matern, U., 1999. Biosynthesis of phenylpropanoids and related compounds. In. Wink, M. (Ed.), Annual plant reviews volume 40: Biochemistry of plant secondary metabolism 2nd Ed. Academic Press, Sheffield, pp. 151-221.

Peterhansel, C., Horst, I., Niessen, M., Blume, C., Kebeish, R., Kürkcüoglu, S., and Kreuzaler, F. 2010. Photorespiration. *The Arabidopsis Book* 8: e0130.1-24.

Pogorelko, G., Lionetti, V., Fursova, O., Sundaram, R.M., Qi, M., Whitham, S.A., Bogdanove, A.J., Bellincampi, D., and Zabotina, O.A. 2013. Arabidopsis and *Brachypodium distachyon* transgenic plants expressing *Aspergillus nidulans* acetylesterases have decreased degree of polysaccharide acetylation and increased resistance to pathogens. *Plant Physiology* 162: 9-23.

Rajkowski, K.T. 2004. Simplified qualitative method for canavanine in seeds and sprouts. *Journal of Food Protection* 67: 212-214.

Ramautar, R., Mayboroda, O.A., Somsen, G.W., and de Jong, G.J. 2011. CE-MS for metabolomics: Developments and applications in the period 2008–2010. *Electrophoresis* 32: 52-65.

Richard, P., and Hilditch, S. 2009. D-Galacturonic acid catabolism in microorganisms and its biotechnological relevance. *Applied Microbiology and Biotechnology* 82: 597-604.

Rochfort, S. 2005. Metabolomics reviewed: A new “omics” platform technology for systems biology and implications for natural product research. *Journal of Natural Products* 68: 1813-1820.

Rodó, P.A, Brugière, N, Vankova, R., Malbeck, J., Olson, J.M., Haines, S.C., Martin, R.C., Habben, J.E., Mok., D.W.S., and Mok, M.C. 2008. Over-expression of a zeatin O-glucosylation gene in maize leads to growth retardation and tasselseed formation. *Journal of Experimental Botany* 59: 2673-2686.

Rodrigues, F.A., Vale, F.X.R., Datnoff, L.E., Prabhu, A.S., and Korndörfer, G.H. 2003. Effect of rice growth stages and silicon on sheath blight development. *Phytopathology* 93: 256-261.

Roessner, U., and Bowne, J. 2009. What is metabolomics all about? *BioTechniques* 46: 363-365

Rojas, C.M., Senthil-Kumar, M., Tzin, V., and Mysore, K.S. 2014. Regulation of primary plant metabolism during plant-pathogen interactions and its contribution to plant defense. *Frontiers in Plant Science* 5: 1-12.

Rosenthal, G.A. 1970. Investigation of canavanine biochemistry in the jack bean plant, *Canavalia ensiformis* (L.) DC. I. Canavanine utilization in the developing plant. *Plant Physiology* 46: 273-276.

Rosenthal, G.A. 1982. L-canavanine metabolism in jack bean *Canavalia ensiformis* (L.) DC. (Leguminosae). *Plant Physiology* 69:1066-1069.

Sakr, N. 2010. Can we enhance durable resistance against *Plasmopara halstedii* (sunflower downy mildew)? *Journal of Plant Protection Research* 50: 15-21.

Salvador, V.H., Lima, R.B., dos Santos, W.D., Soares, A.R., Böhm, P.A.F., Marchiosi, R., Ferrarese, M.L.L., and Filho, O.F. 2013. Cinnamic acid increases lignin production and inhibits soybean root growth. *Plos One* 8: 1-10.

Samborski D.J., and Forsyth F.R. 1960. Inhibition of rust development on detached wheat leaves by metabolites, antimetabolites, and enzyme poisons. *Canadian Journal of Botany* 38: 467-476.

Sana, T.R., Fischer, S., Wohlgemuth, G., Katrekar, A., Jung, K-H., Ronald, P.C., and Fiehn, O. 2010. Metabolomic and transcriptomic analysis of the rice response to the bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae. Metabolomics* 6: 451-456.

Sanchez-Vallet, A., Ramos, B., Bednarek, P., López, G., Piślewska-Bednarek, M., Schulze-Lefert, P., and Molina, A. 2010. Tryptophan-derived secondary metabolites in *Arabidopsis thaliana* confer non-host resistance to necrotrophic *Plectosphaerella cucumerina* fungi. *The Plant Journal* 63: 115–127.

Sato, S., Soga, T., Nishioka, T., and Tomita, M. 2004. Simultaneous determination of the main metabolites in rice leaves using capillary electrophoresis mass spectrometry and capillary electrophoresis diode array detection. *The Plant Journal* 40: 151-163.

Savary, S., Willocquet, L, Elazegui, F.A., Castilla, N.P., and Teng, P.S. 2000. Rice pest constraints in tropical Asia: quantification of yield losses due to rice pests in a

range of production situations. *Plant Disease* 84: 357-369.

Sawada, Y., and Aoki, T. 2014. Metabolomics. In. Tabata, S., Stouugaard, J. (Eds), The *Lotus japonicus* genome. Springer, Heidelberg-Berlin, pp. 171-182.

Seifi, H.S., Bockhaven, J.V., Angenon, G., and Höfte, M. 2013. Glutamate metabolism in plant disease and defense: friend or foe? *Molecular Plant-Microbe Interactions* 26: 475–485.

Shepherd C.J., and Mandryk M. 1964. Effect of metabolites and antimetabolites on the sporulation of *Peronospora tabacina* Adam on tobacco leaf disk. *Australian Journal of Biological Science* 17: 878-891.

Siddiqui, I.A., and Shaukat, S.S. 2002. Phenol-mediated suppression of soil-borne root-infecting fungi in mungbean. *Phytopathologia Mediterranea* 41: 33-38.

Silverman, P., Seskar, M., Kanter, D., Schweizer, P., Métraux, J-P., and Raskin, I. 1995. Salicylic Acid in rice, biosynthesis, conjugation, and possible role. *Plant Physiology* 108: 633-639.

Simonetta, S., Avidano, L., and Berta, G. 2007. Morphogenetic effects induced by pathogenic and non pathogenic *Rhizoctonia solani* Kühn strains on tomato roots. *Caryologia* 60: 141-145.

Simola L.K., and Lönnroth K. 1979. The effect of some protein and non-protein amino acid on the growth of *Cladosporium herbarum* and *Trichothecium roseum. Physiologia Plantarum* 46: 381-387.

Singh, S., Asthir, B., Bains, N.S., and Mann, S. K. 2009. Induction of carbohydrate metabolism in relation to leaf blight in barley (*Hordeum vulgare*). *Advances in Biological Research* 3: 61-66.

Slaton, N.A., Cartwright, R.D., Meng, J., Gbur, E.E., and Norman R.J. 2003. Sheath blight severity and rice yield as affected by nitrogen fertilizer rate, application method, and fungicide. *Agronomy Journal* 95: 1489-1496.

Smith, P.M.C., and Atkins, C.A. 2002. Purine biosynthesis. big in cell division, even bigger in nitrogen assimilation. *Plant Physiology* 128:793-802.

Soga, T., Baran, R., Suematsu, M., Ueno, Y., Ikeda, S., Sakurakawa, T., Kakazu, Y., Ishikawa, T., Robert, M., Nishioka, T., and Tomita, M. 2006. Differential metabolomics reveals ophthalmic acid as an oxidative stress biomarker indicating hepatic glutathione consumption. *Journal of Biological Chemistry* 281: 16768

-16776.

Soga, T., Igarashi, K., Ito, C., Mizobuchi, K., Zimmermann, H-P., and Tomita, M. 2009. Metabolomic profiling of anionic metabolites by capillary electrophoresis mass spectrometry. *Analytical Chemistry* 81: 6165-6174.

Soga, T., Ohashi, Y., Ueno, Y., Naraoka, H., Tomita, M., and Nishioka, T. 2003. Quantitative metabolome analysis using capillary electrophoresis mass spectrometry. *Journal of Proteome Research* 2: 488-494.

Staub, A., Schappier, J., Rudaz, S., and Veuthey, J-L. 2009. CE-TOF/MS: fundamental concepts, instrumental considerations and applications. *Electrophoresis* 30: 1610-1623.

Sugimoto, M., Kawakami, M., Robert, M., Soga, M., and Tomita, M. 2014. Bioinformatics tools for mass spectroscopy-based metabolomic data processing and analysis. *Current Bioinformatics* 7: 96-108.

Sugimoto, M. Wong, D.T., Hirayama, A., Soga, T., and Tomita, M. 2010. Capillary electrophoresis mass spectrometry-based saliva metabolomics identified oral, breast and pancreatic cancer-specific profiles. *Metabolomics* 6: 78-95.

Suharti, W.S., Nose, A., and Zheng, S-H. 2016. Metabolite properties of sheath blight disease resistance in rice: in the case of positive ion mode analysis by CE/TOF-MS. *Plant Production Science* 19: 279-290.

Sumner, L.W., Mendes, P., and Dixon, R.A. 2003. Plant metabolomics: large-scale phytochemistry in the functional genomics era. *Phytochemistry* 62: 817–836.

Sundravadana, S., Alice, D., Kuttalam, S., and Samiyappan, R. 2007. Azoxystrobin activity on *Rhizoctonia solani* and its efficacy against rice sheath blight. *Tunisian Journal of Plant Protection* 2: 79-84.

Suzuki, N., Miller, G., Salazar, C., Mondal, H.A., Shulaev, E., Cortes, D.F., Shuman, J.L., Luo, X., Shah, J., Schlauch, K., Shulaev, V., and Mittler, R. 2013. Temporal-spatial interaction between reactive oxygen species and abscisic acid regulates rapid systemic acclimation in plants. *The Plant Cell* 25: 3553–3569.

Taheri, P., and Tarighi, S. 2010. Riboflavin induces resistance in rice against Rhizoctonia solani via jasmonate-mediated priming of phenylpropanoid pathway. *Journal of Plant Physiology* 167: 201-208.

Taheri, P., and Tarighi, S. 2011. Cytomolecular, aspects of rice sheath blight caused by *Rhizoctonia solani. European Journal of Plant Pathology* 129: 511-528.

Takahashi, H., Matsumura, H., Kawai-Yamada, M., and Uchimiya, H. 2008. The cell death factor, cell wall elicitor of rice blast fungus (*Magnaporthe grisea*) causes metabolic alterations including GABA shunt in rice cultured cells. *Plant Signaling Behavior* 3: 945-953.

Takahashi, N., and Washio, J. 2011. Metabolomic effects of xylitol and fluoride on plaque biofilm in vivo. *Journal of Dental Research* 90: 1463-1468.

Takei, K., Takahashi, T., Sugiyama, T., Yamaya, T., and Sakakibara, H. 2002. Multiple routes communicating nitrogen availability from roots to shoots: a signal transduction pathway mediated by cytokinin. *Journal of Experimental Botany* 53: 971-977.

Tauzin, A.S., and Giardina, T. 2014. Sucrose and invertases, a part of the plant defense response to the biotic stresses. *Frontiers in Plant Science* 5:1-8.

Tavernier, V., Cadiou, S., Pageau, K., Lauge´, R., Reisdorf-Cren, M., Langin, T., and Masclaux-Daubresse, C. 2007. The plant nitrogen mobilization promoted by *Colletotrichum lindemuthianum* in phaseolus leaves depends on fungus pathogenicity. *Journal of Experimental Botany* 58: 3351-3360.

Tohge, T., Nishiyama, Y., Hirai, M.Y., Yano, M., Nakajima, J., Awazuhara, M., Inoue, E., Takahashi, H., Goodenowe, D.B., Kitayama, M., Noji, M., Yamazaki, M., and Saito, K. 2005. Functional genomics by integrated analysis of metabolome and transcriptome of Arabidopsis plants over-expressing an MYB transcription factor. *Plant Journal* 42: 218-235.

Tran, D. V. 1997. World rice production: main issues and technical possibilities. In. Chataigner, J. (Ed.). Activités de recherche sur le riz en climat méditerranéen. CIHEAM, Montpellier, pp. 57-69.

Tzin, V., and Galili, G. 2010. The biosynthetic pathways for shikimate and aromatic amino acids in *Arabidopsis thaliana*. *The Arabidopsis Book* 8: e0132.1-18.

Vance, C.P. 1980. Lignification as a mechanism of disease resistance. *Annual Review of Phytopathology* 18: 259-288.

Van Der Plank, J.E. 1966. Horizontal (polygenic) and vertical (oligogenic) resistance against blight. *American Potato Journal* 43: 43-52.

Veit, S., Wörle, J.M., Nürnberger, T., Koch, W., and Seitz H.U. 2001. Novel protein elicitor (PaNie) from *Pythium aphanidermatum* induces multiple defense responses in carrot, arabidopsis, and tobacco. *Plant Physiology* 127: 832-841.

Vogel-Adghough, D., Stahl, E., Návarová, H., and Zeier, J. 2013. Pipecolic acid enhances resistance to bacterial infection and primes salicylic acid and nicotine accumulation in tobacco. *Plant Signaling Behavior* 8: 1-9.

Voss, I., Sunil., B., Scheibe, R., and Raghavendra, A.S. 2013. Emerging concept for the role of photorespiration as an important part of abiotic stress response. *Plant Biology* 15: 713-722.

Wakasa, K., and Ishihara, A. 2009. Metabolic engineering of the tryptophan and phenylalanine biosynthetic pathways in rice. *Plant Biotechnology* 26: 523–533.

Wang, Y., Xu, L., Shen, H., Wang J., Liu, W., Zhu, X., Wang, R., Sun, X., and Liu, L. 2015. Metabolomic analysis with GC-MS to reveal potential metabolites and biological pathways involved in Pb and Cd stress response of radish roots. *Scientific Reports* 5: 18296.1-13

Wasano, K., and Dhanapala, M. 1982. Genetic analysis and selection for polygenic resistance to bacterial leaf blight (*Xanthomonas campestris* pv. *oryzae*) of rice. *Japanese Journal of Tropical Agriculture* 26: 130-139.

Wasano, K., and Hirota, Y. 1986. Varietal differences in the resistance to sheath blight disease caused by *Rhizoctonia solani* Kuhn, by the syringe inoculation methods. *Bulletins of the Faculty of Agriculture of Saga University* 60: 49-59.

Wasano, K., Hirota, Y., and Kido, Y. 1985. Varietal differences in the resistance to sheath blight of rice, *Rhizoctonia solani* Kuhn, and effectiveness of selection for the resistance from the cross, Tetep x CN4-4-2 *Report of the Kyushu Branch of the Crop Science Society of Japan* 52: 16-22*.

Wasano, K., Oro, S., and Kido, Y. 1983. The syringe inoculation method for selecting rice plants resistant to sheath blight, *Rhizoctonia solani* Kühn. *Japanese Journal of Tropical Agriculture* 27: 131-134.

Wink, M. 1988. Plant breeding: importance of plant secondary metabolites for protection against pathogens and herbivores. *Theoretical and Applied Genetics* 75: 225-233.

Winter, G., Todd, C.D., Trovato, M., Forlani, G., and Funck, D. 2015. Physiological implication of arginine metabolism in plants. *Frontiers in Plant Science* 6: 1-14.

Wisser, R.J., Sun, Q., Hulbert, S.H., Kresovich, S., and Nelson, R.J. 2005. Identification and characterization of regions of the rice genome associate with broad-spectrum, quantitative disease resistance. *Genetics* 169: 2277-2293.

Wojciecchowska, E., Weinert, C.H., Egert, B., Trierweiler B., Schmidt-Heydt, M., Horneburg, B., Graeff-Hönninger, S., Kulling, S.E., and Geisen, R. 2014. Chlorogenic acid, a metabolite identified by untargeted metabolome analysis in resistant tomatoes, inhibits the colonization by *Alternaria alternata* by inhibiting alternariol biosynthesis. *European Journal of Plant Pathology* 139: 736-747.

Wolfender, J-L., Marti, G., Thomas, A., and Bertrand, S. 2015. Current approaches and challenges for the metabolite profiling of comple natural extract. *Journal of Chromatography* 1382:136-164.

Yang, D., Du, X., Yang, Z., Liang, Z., Guo, Z., and Liu, Y. 2014. Transcriptomics, proteomics, and metabolomics to reveal mechanisms underlying plant secondary metabolism. *Engineering in Life Sciences* 14: 456-466.

Yemm, E.W., Cocking, E.C., and Ricketts, R.E. 1955. The determination of amino acids with nynhidrin. *Analyst* 80: 209-214.

You, L., Zhang, B., and Tang, Y.J. 2014. Application of stable isotope-assisted metabolomics for cell metabolism studies. *Metabolites* 4: 142-165.

Yadav, S., Anuradha, G., Kumar, R.R., Vemireddy, L.R., Sudhakar, R., Donempudi, K., Venkata, D., Jabeen, F., Narasimhan, Y.K., Marathi, B., and Siddiq, E.A., 2015. Identification of QTLs and possible candidate genes conferring sheath blight resistance in rice (*Oryza sativa* L.). *Springerplus* 175: 1-12.

Yellareddygari, S.K.R., Reddy, M.S., Kloepper, J.W., Lawrence, K.S., and Fadamiro, H. 2014. Rice sheath blight: a review of disease and pathogen management approaches. *Journal of Plant Pathology & Microbiology* 5: 1000241.2-4

Zeng, Y., Ji, Z., Ma, L., Li, X., and Yang, C. 2011. Advances in mapping loci conferring resistance to sheath blight and mining *Rhizoctonia solani* resistance resources. *Rice Science* 18: 56-66.

Zou, J.H., Pan, X.B., Chen, Z.X., Xu, J.Y., Lu, J.F., Zhai, W.X., and Zhu, L.H. 2000. Mapping quantitative trait loci controlling sheath blight resistance in two rice cultivars (*Oryza sativa* L.). *Theoretical and Applied Genetics* 10: 569-573.

Zuo, S-M., Zhang, Y-F., Chen, Z-X., Jiang, W., Feng, M-H., and Pan, X-B. 2014. Improvement of rice resistance to sheath blight by pyramiding QTLs conditioning disease resistance and tiller angle. *Rice Science* 21: 318−326.

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