INTRODUCTION

Nematodes are the major pathogens that caused yield losses in tomato plants worldwide. The nematode is one of the obligate parasites which infect a huge number of plants and impact the loosing of production (Hu et al., 2015). According to Sasser & Freckman (1987) nematode attacked tomato and the impact reduced production until 20.20% per year. Generally, nematode attacks the plant by sucking the root cells, interfering with the vessel tissues, inhibiting the water, and nutrient translocations. Therefore, this parasite affects the process of photosynthesis and transpiration of the plants. Melakeberhan, Webster, Brooke, D'Auria, & Cackette (1987) also explained that nematode infection could inhibit plant growth, nutrient translocation, affect nutrient deficiency, and cause yield losses (Melakeberhan, Webster, Brooke, D'Auria, & Cackette, 1987).

Many methods can be used to control the nematode attack, however Jacquet et al. (2005) described that using the resistant variety is the most effective way to control the nematode, reduce the cost and minimize environment residues. Plant resistance to nematode has the R gene that produces phenolic compounds including caffeic acid which has been studied previously with the negative effects on the development of nematodes (Ohri & Pannu, 2010). In addition to the presence of phenolic compounds, it is suspected that the resistance tomato has the high content of sugar compounds. According to Eloh, Sasanelli, Maxia, & Caboni (2016), sugar compounds are the signals that are important to the immune response of the tomato plant defense mechanism to the nematode.

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* Corresponding author:
E-mail: eniknurlaili21@ugm.ac.id

ABSTRACT

The resistant tomato against nematode produces various biochemical compounds associated with the defense mechanisms for nematode attack. These compounds allegedly expressed in all cells not only in the infected area. These metabolites are useful for plant development program to point out the candidate traits based on specific metabolites. This study aimed to compare expression of metabolomics as defense mechanism in root and leaf of the plant. Four cultivars consisted of the resistant [GM2 and F1 (a cross GM2 and Hawaii 7996)] and susceptible (Gondol Putih and Gondol Hijau) were used as plant materials. Fifty mg of freeze-dried of root and leaf were taken for assessing 1H-NMR (Nuclear Magnetic Resonance) for metabolomic analysis. Tomato roots resulted in 16 metabolites, while in the leaf detected 15, including amino acid, sugar, and aromatic compounds. Both root and leaf showed the same metabolites that play an important role in nematode-resistant mechanism, these metabolites were α- and β-glucose, and caffeic acid. These compounds had the same concentration within the root and leaf. It is implied that the metabolites associated with defense mechanism of tomato plants against nematode not only expressed locally in the infected area but also produced by not infected tissues throughout the plant.
Previous studies have reported the compounds that associated with plant resistance are expressed thoroughly in all the plant cells. Pieterse & van Loon (1999) point out that activation genes that associated with plant defense against pathogens expressed systemically. It provides broad resistance spectrum of the viruses, bacteria, fungi and other pathogens in both infected and non-infected plants. Eloh, Sasanelli, Maxia, & Caboni (2016) compared the metabolic changes between tomato leaves and stems due to nematode attack in susceptible plant, unfortunately they did not analyze the differences compounds associated with tomato plant resistance and susceptible plant in the leaf and root (infected area). According to Park & Ryu (2015), the whitefly which attacks the leaves of tomato plants produced an elicitor that triggers the production of salicylic acid and jasmonic acid that were expressed on the leaves and roots of the plants.

Kaplan et al. (2008) explained that the study about variation expression in plant defense mechanism against root-knot nematode in the above and under part of plants is still not widely recognized because root had more contact with nematode penetration, more direct and firstly recognize with nematode penetration. Therefore, many studies in root-knot nematode just observed in the root expression. Further study is needed to unravel the different metabolite between root and leaves that associated with resistance to nematodes. Comparison of the different metabolite between root and leaf needs some technologies that can detect the smallest compounds that associated in tomato defense mechanism to root-knot nematode.

The metabolomic approach is an effective study that can detect the total metabolic of biological systems in plants as result of interactions between pathogens and plants (Kim, Choi, & Verpoorte, 2010) and as a response within plants and the environment (Allwood, Ellis, & Goodacre, 2008). These compounds will be guiding researchers to point out the gene candidates based on the specific metabolites for improving plant quality. Fernandez et al. (2016) explained that metabolomic used as a biomarker because associated with genotypes and belongs to the phenotypes.

There is no information of comparison the metabolites expression in the root and leaf of the nematode attacked plants. This paper elaborated the metabolites associated with resistance that expressed in leaf and root of the resistant and susceptible tomato.

**MATERIALS AND METHODS**

**Procedures**

This study was conducted from October 2016 to June 2017 in the greenhouse of Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta, Indonesia. Tomato cultivars consisted of a resistance (GM2), medium resistant (F1: GM2 x Hawaii 7996) and susceptible (Gondol Putih and Gondol Hijau) were used in this study. Seeds were sown in the sterile soil medium consisted of NPK (1:1:1) at a ratio of 1:50 (w/w). Three weeks after sowing, the seedlings were transplanted into polybags (130 x 130 mm) individually. There are two treatments consisted of a control (not inoculated with nematode) and nematode inoculation (± 2000 juveniles II per plant) applied to all of the plants at one week after transplanting. Each cultivar and treatment had five replications. In the 45 days after transplanting the plant was harvested and collected for metabolomics analysis. The sample of root and leaves were collected approximately 5 g per plant for NMR analysis.

**Metabolomic Analysis Using ¹H-NMR**

The metabolomic analysis of this experiment based on López-Gresa et al. (2010). ¹H-NMR (Nuclear Magnetic Resonance) was used to detect the metabolic profile of samples. After harvesting samples, leaf and root cleaned and wash with distilled water. Approximately 5 g samples per plant ground in the mortar with liquid nitrogen, then placed in the 5 ml tube for freeze-dried treatment for about 48 hours. Fifty mg freeze-dried samples were taken into a 2 ml tube mixed with 0.05% internal standard (trimethyl silyl-3-propionic acid/TMSP) and 1 ml of CD$_3$OD. Then 20 minutes vortexed to mix the solution, then placed in the sonication bath about 20 minutes, and centrifuged with 13,000 ppm for 10 minutes. ~800 µl of root and leaf suspension was placed in the NMR tube for ¹H-NMR analysis using a 500 MHz Jeol NMR spectrometer. At 30°C the spectra were recorded with 26 s acquisition time and 128 scans for 10 minutes (0.16 Hz per data point, 30 pulse width 11.3 µs, and 1.5-second relaxation delay).
Data Analysis
Signals resulted from NMR analysis were identified using MNOVA software. Spectral intensities were scaled to total intensity. Spectra were phased and baseline manually and then were calibrated to TMSP by setting at 0.0 ppm. Undesired signals were excluded by removing residual water and methanol. Detection of metabolites based on metabolomics data from previous studies such as Escudero, Marhuenda-Egea, Ibáñez-Cañete, Zavala-Gonzalez, & Lopez-Llorca (2014), López-Gresa et al. (2010), and Romero González (2011). Principal Component Analysis/PCA and OPLS-DA Orthogonal Projections to Latent Structures Discriminant Analysis were used to interpret the multivariate data using SIMCA-P software. Before analysis, the data were scaled to Pareto and validated by permutation analysis and CV-ANOVA. Data collected from a semi-quantitative analysis performed using statistic software analysis. Varian of the data homogenized then tested with Tukey's test at 5%.

RESULTS AND DISCUSSION
Leaf and Root Metabolites
This study compared metabolomics between tomato root and leaf to find the location of metabolites produced by resistant tomato plants against nematode. There were two treatments in this study including a control (not inoculated) and inoculated with root-knot nematode to reveal that these metabolites may be expressed constitutively or inducible. A study of Lanubile et al. (2014) analyzed the resistant genotypes of maize between control and infected with *F. verticillioides* to study the functional genomic analysis of constitutive and inducible defense response against *F. verticillioides*. Based on the 1H-NMR signal resulted from the root and leaf tomato sample included not inoculated and inoculated with a root-knot nematode (Fig. 1) could be divided into three regions: Amino acid groups, sugar, and aromatic groups. Metabolites were detected using semi-quantitative analysis based on the metabolomics data of tomato. Fig. 1 showed that many signals were detected in the tomato root and leaf, but not all of the data could be identified. Table 1 described that in the root sample resulted in 16 metabolites and 15 metabolites from the leaf samples. This data compared with the previous study of Afifah (2018) and Afifah, Murti, & Nuringtyas (2019) resulted in root metabolomics data. The data categorized into the amino acid groups, sugar, and organic acid. Leucine, valine, alanine, acetic acid, succinate, GABA, ethanolamine, choline, glycine, β- and α-glucose, caffeic acid, fumaric acid, PAL, UDPG were found in the leaf of tomato plants. The roots have been identified for leucine, valine, alanine, acetic acid, succinate, GABA, ethanolamine, choline, glycine, β- and α-glucose, caffeic acid, fumaric acid, PAL, UDPG and formate acid.
Table 1. Identification profile metabolites using $^1$H-NMR (in MEOD$_4$)

| No. | Metabolic         | Chemical shift (ppm) and coupling constants (Hz) |
|-----|-------------------|-----------------------------------------------|
|     |                   | Leaf            | Root            |
| 1   | Leucine           | 0.93 (d, $J = 0.7$ Hz)   | 0.94 (d, $J = 0.7$ Hz)   |
| 2   | Valine            | 1.01 (d, $J = 7.0$ Hz); 1.05 (d, $J = 7.0$ Hz); 1.05 (d, $J = 7.0$ Hz) | 1.00 (d, $J = 7.0$ Hz)   |
| 3   | Alanine           | 1.45 (d, $J = 7.2$ Hz)   | 1.45 (d, $J = 7.2$ Hz)   |
| 4   | Acetic acid       | 1.97 (s)         | 1.95 (s)         |
| 5   | Succinate         | 2.54 (s)         | 2.52 (s)         |
| 6   | GABA (γ-aminobutyric acid) | 1.88 (m); 2.34 (t, $J = 7.2$ Hz); 2.23; 3.01 (t, $J = 7.08$ Hz) | 1.88 (m); 2.34 (t, $J = 7.2$ Hz); 2.23; 2.96 (t, $J = 7.08$ Hz) |
| 7   | Ethanolamine      | 3.11 (t, $J = 5.5$ Hz)   | 3.11 (t, $J = 5.5$ Hz)   |
| 8   | Choline           | 3.2 (s)          | 3.2 (s)          |
| 9   | Glycine           | 3.5 (s)          | 3.5 (s)          |
| 10  | β-glucose         | 4.47 (d, $J = 7.8$ Hz)   | 4.46 (d, $J = 7.8$ Hz)   |
| 11  | α-glucose         | 5.09 (d, $J = 3.76$ Hz)   | 5.09 (d, $J = 3.76$ Hz)   |
| 12  | Caffeic acid      | 6.35 (d, $J = 16.0$ Hz); 6.76 (d, $J = 8.2$ Hz); 7.05 (dd, $J = 8.2$, 2.0 Hz); 7.18 (d, $J = 2.0$ Hz); 7.59 (d, $J = 15.9$ Hz) | 6.35 (d, $J = 16.0$ Hz); 6.8 (d, $J = 8.2$ Hz); 7.05 (dd, $J = 8.2, 2.0$ Hz); 7.18 (d, $J = 2.0$ Hz); 7.59 (d, $J = 15.9$ Hz) |
| 13  | Fumaric acid      | 6.53 (s)         | 6.66 (s)         |
| 14  | PAL (Phenylalanine) | 7.33 (d, $J = 8.0$ Hz); 7.39 (d, $J = 8.0$ Hz) | 7.31 (d, $J = 8.0$ Hz); 7.39 (d, $J = 8.0$ Hz) |
| 15  | UDPG (Uridine dphospoglucose) | 8.00 (d, $J = 8.0$ Hz) | 8.00 (d, $J = 8.0$ Hz) |
| 16  | Formate acid      | -                | 8.5 (s)          |

Remarks: $J$ = The coupling constant; d = doublet; dd = a doublet of doublets; m = complex multiple; s = singlet; t = triplet

Statistical Analysis

Firstly, the metabolite profiles were performed using PCA multivariate data analysis but only resulted in 23% data variation (from leaf data) and 22% (from the root data), therefore this data processed thoroughly using OPLS-DA and performed 68% data variation with $R^2$ value = 0.98, $Q^2 = 0.79$, validated with CV-ANOVA resulted in $F$ value = 3.6, df = 23 (from leaf data). Root data resulted 42% variation with $R^2$ value = 0.989, $Q^2 = 0.549$ and then validated with CV-ANOVA resulted $F$ value = 0.43, df = 23, and $P = 0.02$. This analysis indicated a good model because has a $Q^2$ value of more than 0.5 (Leiss, Choi, Abdel-Farid, Verpoorte, & Klinkhamer, 2009). Based on the score plot (Fig. 2a) in the leaf and root data (Fig. 2b) showed that resistant cultivars separated significantly from the susceptible cultivars. Based on the loading plot both in the leaf and root data (Fig. 2c and Fig. 2d) showed that separation metabolites caused by the concentration of α- and β-glucose, and caffeic acid. Based on the score plot and loading plot, these metabolites located in the same quadrant with the resistant cultivars. This result following the previous study (Afifah, Murti, & Nuringtyas, 2019) that these compounds differentiated between resistant and susceptible plants.

Tomato root and leaf metabolites in the non-inoculated and inoculated treatments showed the same information (Fig. 3), the resistant cultivars (GM2) had a higher value of α-glucose, then followed by medium resistant (F1) and the lowest were susceptible plants. In the root data, there was no significant difference in α-glucose concentration between GM2 and F1, whereas the leaf sample had a significant difference.
Fig. 2. Root score plot (a), leaf score plot (b), root loading plot (c), and leaf loading plot (d) resulted from OPLS-DA. The different colors and values in the loading plot described the chemical shift (ppm) from a bucket of $^1$H-NMR data.

Fig. 3. The concentration of $\alpha$-glucose.

Remarks: Bars with the same letter in each group were not significantly different at $P = 0.05$ based on Tukey’s HSD tests.
Study of Murti, Muamiroh, Pujiat, & Indarti (2012), GM2 had a high resistance to nematode based on the percentage of root gall number after nematode infection and could inhibit nematode development. It had the highest metabolite associated with the resistance mechanism to the nematode. F1 cultivar resulted from a crossing between GM2 and Hawaii 7996, therefore F1 had a medium resistant to the nematode. α-glucose found between infected and non-infected cultivars, it is indicated this metabolite produced constitutively before and after nematode infection.

β-glucose is one of the metabolites also found between tomato root and leaf and alleged as a compound that differentiated between resistant and susceptible cultivars (Fig. 4). This compound also produced by non-infected and infected tomato plants. Both root and leaf gave the same meaning that GM2 had a higher value of β-glucose then followed by F1 and the lowest was susceptible cultivars. β-glucose showed an increase after nematode infection in the root, but tomato leaf had a downtrend of β-glucose after nematode infection in the GM2 cultivars. β-glucose is a primary metabolite, an important compound for plant metabolism resources and other regulations. Plant responses to biotic and abiotic are very complex phenomena, therefore different stress in each tissue of the plant responses to different sugar sensing mechanisms (Rosa et al., 2009).

Caffeic acid, a biochemical compound that also differentiated between resistant and susceptible cultivars was found in the tomato root and leaf. Fig. 5 showed only GM2 had a higher value of caffeic acid, F1 had no different with susceptible cultivars. Caffeic acid showed an increase after nematode infection, therefore, GM2 in the tomato root and leaf data with inoculated treatment had a higher of caffeic acid than non-inoculated treatments. It is indicated that caffeic acid, a metabolite that differentiated between resistant and susceptible tomato plants produced in the root and leaf tissue, expressed systematically under and above part of the plants. The study of Kaplan et al. (2008) about Nicotiana resistant to root-knot nematode resulted in secondary metabolites were produced in the root part and then translocated in the above part of the plants. This mechanism is a response of the plants to protect themselves against herbivory.

![Graph](https://via.placeholder.com/150)

Remarks: Bars with the same letter in each group were not significantly different at P = 0.05 based on Tukey's HSD tests

**Fig. 4.** The concentration of β-glucose
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Remarks: Bars with the same letter in each group were not significantly different at P = 0.05 based on Tukey’s HSD tests.

**Fig. 5.** The concentration of Caffeic acid

Three metabolites found in this study (α- and β-glucose and caffeic acid) were expressed in the tomato root and leaf constitutively. It is suggested that metabolomics associated with a defense mechanism against nematode expressed systemically (both of root and leaf tissue) and constitutively (before and after nematode infection). Based on the study of Rasmann & Agrawal (2008), in Solanaceous species, the chemical compounds associated with defense mechanisms are synthesized in the root and then transported into the above part of the plants. Plants attacked by above- and underground pathogens may produce systemic responses against pathogens. The infected area of the plant can trigger stress signaling then transported through all parts of the plant systemically (Kammerhofer et al., 2015).

When infecting the plant, nematode affects the root tissue of the plants, changes metabolism, gene expression involved in defense mechanism, nutrient, and biosynthesis and signaling of phytohormone. It could be shown that the systemic effect in aboveground plant tissue can be associated with increasing several levels of biochemical signals including JA, SA, and other hormones (Wubben, Jin, & Baum, 2008). Manosalva et al. (2015), the induction of chemical compounds (ascarosides) produced by nematode to attack the plants can increase the resistant level to *P. infestans*. The Arabidopsis root that applicated with ascarosides can decrease the population of *P. infestans* on the root. Islam, Mercer, Leung, Dijkwel, & McManus (2015) described that jasmonic acid (JA) signaling genes that corresponded to nematode attack expressed systemically and locally in the root, gene Tr-ACTS1 expressed locally. It suppressed ET productions that are important for root galling in the plant. This finding may guide the researchers to point out the gene candidates for nematode-resistant based on the metabolites associated with the defense mechanism because metabolomics is derived from genetics and associate with the phenotypes. This information also useful for taking samples for metabolomics samples for tomato-nematode resistant analysis, whereas root located in the bottom of the plant, needs extra treatment than leaf samples for cleaning and crushing.

**CONCLUSION**

Metabolomics data were identified between root and leaf with the same data interpretations. Sixteen metabolites were found from leaf and 15 from root samples. Caffeic acid, α- and β-glucose were systemically expressed in the root and leaf of tomato plants with the same concentration. These metabolites allegedly have an important role in the
resistant mechanism to nematode and expressed through the plant.

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