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The Genes Associated with Jasmonic Acid and Salicylic Acid Are Induced in Tropical Chili Pepper against *Ralstonia solanacearum* by Applying Arbuscular Mycorrhizal Fungi

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Abstract: Controlling soil-borne pathogens is a significant problem in agriculture. Arbuscular mycorrhizae have a potential role in controlling soil-borne pathogens by increasing plant phytohormone contents. However, the mechanism of resistance by mycorrhizae has not been fully elucidated, particularly against bacterial wilt disease in Solanaceae. This study examined the role of mycorrhizae in expressing genes involved in the signaling pathways mediated by jasmonic acid (JA) and salicylic acid (SA) in tropical chili pepper against the bacterium *Ralstonia solanacearum*. Seedlings from ten genotypes of chili pepper were inoculated with a consortium of five mycorrhizal species and/or inoculated with a mixture of nine isolates of *R. solanacearum*. The leaves of 10-week-old plants after the treatment were sampled for real-time polymerase chain reaction analysis. The results showed that the mycorrhizae strengthened the immune system of tropical chili pepper by increasing the relative gene expression levels of JA and SA in genotypes with high and low responsiveness to the mycorrhizae. The relative gene expression level of JA was related to the percentage colonization of mycorrhizae and the resistance of the tropical chili pepper genotypes to *R. solanacearum*. The relative gene expression level of SA was associated with the resistance of tropical chili pepper to *R. solanacearum*.

Keywords: resistance induction; jasmonic acid; salicylic acid; tropical chili pepper; *Ralstonia solanacearum*; arbuscular mycorrhiza

1. Introduction

Pepper and sweet pepper (*Capsicum* spp.) have many uses as spices, natural food coloring agents, and raw materials for making medicines and pesticide mixtures [1–4]. One of the obstacles to chili cultivation during the rainy season is the systemic vascular wilt disease caused by *Ralstonia solanacearum*. *R. solanacearum* is a most devastating soil-borne pathogen, particularly in the Solanaceae family [5–12]. The bacterial wilt pathogen *R. solanacearum* remains difficult to control because of its persistence. This pathogen can survive on plant debris or in the absence of a host. It has a broad host range, high genetic diversity, complex sub-species, a broad geographic distribution, and lives and multiplies in the xylem, thereby blocking the xylem tissue [5,7,13–18].

Current management of pathogens is based on chemical pesticides. However, using chemical pesticides produces residues in crops that are harmful to health, impact the environment, and result in an unsustainable agricultural system [5,19,20]. In recent years, the

specific microbiome has been recognized as a potential tool for achieving environmentally friendly agriculture. The importance of the microbiome as a modulator of crop resistance has emerged [5,19,21–24]. The presence of certain microorganisms induces the reprogramming of plant metabolic pathways involved in the defense system and increases the ability of plants to survive under adverse conditions [5,19,23–25].

Endophytic fungi, such as arbuscular mycorrhizae, have potential roles in controlling plant diseases, particularly soil-borne pathogens [26–28]. Local and systemic mycorrhizal-induced resistance (MIR) increases phytohormone contents, such as salicylic acid (SA) and jasmonic acid (JA) [23,24,29–31]. The response of plants to biotrophic pathogens is generally controlled by activating a salicylic acid (SA) dependent response [32]. Beneficial microorganisms modulate the SA pathway [19]. Activating the SA pathway may also influence the favorable performance of the symbiont.

JA is another essential hormone in plant defense that regulates various processes related to plant development, symbiosis, and plant responses to insects and pathogens [32–34]. JA stimulates induced systemic resistance (ISR) activated by the interactions between plants and certain beneficial microorganisms. Antagonism of the JA and SA biosynthetic pathways occurs [35]; however, some researchers have reported synergistic interactions between them [36,37]. Modulation of the JA signaling pathway may be involved in the symbiosis by keeping the endophytic fungi in the asymptomatic stage [31,38].

Managing beneficial microbes for plants has been proposed as a new platform to revolutionize plant protection against pathogens. However, the mechanisms underlying the symbiotic relationship between plants and beneficial microorganisms are still being investigated. Several studies have shown that biocontrol agents efficiently reduce the incidence of bacterial wilt disease in greenhouses, but field trials have not yielded results [5].

The present study examined the symbiotic relationship between tropical chili pepper genotypes and mycorrhizae. Genotypes of peppers with different responsiveness to mycorrhizae and different resistance to bacterial wilt disease were studied. This study aimed to investigate the potential of mycorrhizae to increase the resistance of the tropical chili pepper genotypes against *R. solanacearum* through hormone signaling pathways. The potential of mycorrhizae was studied in relation to the relative expression of the genes associated with JA and SA signaling pathways. The effect of mycorrhizae on phenotypic traits of the tropical chili pepper was also investigated.

2. Materials and Methods

2.1. Chili Pepper Genotypes, Mycorrhizae, and the *Ralstonia solanacearum* Isolates

This study was carried out at the Agrotechnology Innovation Center of the Universitas Gadjah Mada at Kalitirto Yogyakarta, Indonesia (7°79'58.59" N, 110°46'52.85" E). The experiment examined the relative gene expression of JA and SA at the generative growth phase when the plant started flowering, of ten tropical chili pepper genotypes with different responses to mycorrhizae and resistance to *R. solanacearum*.

Ten chili pepper genotypes were used in this study. The genotypes consisted of eight accessions from the Genebank of the Universitas Gadjah Mada and East-West Seed Indonesia (Ewindo) and two commercial cultivars. Based on the published descriptions of the cultivars, the two commercial cultivars were used as the resistant check cultivar (C-50, Known You Seed) and the susceptible check cultivar (C-54, Oriental Seed) against *R. solanacearum*. The mycorrhizal inoculum was a mixture of *Glomus* sp., *Funneliformis* sp., *Acaulospora* sp., *Gigaspora* sp., and *Scutellospora* sp. with a zeolite carrier obtained from the Agricultural Microbiology Laboratory, Faculty of Agriculture, Universitas Gadjah Mada. Mycorrhizal content was 500 inoculums per gram of zeolite carrier. The *R. solanacearum* isolate used was a mixture of nine bacterial isolates.

2.2. Procedure of Experiments

The study consisted of three experiments in parallel using separate sets of experimental plants.

Experiment 1a. Evaluation of genotypes responsiveness to mycorrhizae

The seeds of the ten chili pepper genotypes were germinated in a germination box for 5 days. Twenty seedlings of each genotype were transplanted to nursery pot trays filled with sterilized growing media. The medium was a mixture of soil, coco peat, and husk charcoal at a ratio of 2:1:1 (*v:v:v*). Before transplanting the seedlings, the medium was mixed with the mycorrhizal inoculum at a ratio of 10:1 (*w:w*). The seedlings were maintained in the greenhouse until they were 21 days old.

The 21-day-old seedlings were transplanted into a 10-L planter bag filled with 7 kg of planting media per planter bag. The planting media was a mixture of soil, compost, coco peat, and husk charcoal at a ratio of 2:1:1:1 (*v:v:v:v*). The plants were maintained in the screen house until 10 weeks old.

The growth response to mycorrhizal symbiosis was defined as an increase in plant biomass inoculated with mycorrhiza compared to the biomass of plants that were not inoculated [39]. The plants were sampled 10 weeks after transplant to examine the dry biomass. The plant biomass was represented by shoot and root dry weight. Dry shoots and roots were weighed after the biomass was sun-dried in a greenhouse for seven days and oven-baked at 68 °C for 48 h. The percentage of mycorrhizal colonization was determined by staining the roots using trypan blue 0.05% (*w:v*) as described in the previous study [40] with modifications. The percentage of mycorrhizal colonization was calculated from the proportion of the mycorrhizal root area to the total root area. The area was measured using the Fiji program [41].

Experiment 1b. Evaluation of genotypes resistance to *R. solanacearum*

The seeding procedure was conducted similarly to Experiment 1a. Shortly after the seedlings were transplanted into the nursery pot trays, the *R. solanacearum* was inoculated using the drenching method. A mixture of nine strains of the *R. solanacearum* bacterial suspension at a concentration of 1×10^8 CFU mL⁻¹ (OD600 = 0.1) was poured (20 mL of each) into the planting medium around the root area.

The resistance of the ten tropical chili pepper genotypes to *R. solanacearum* was evaluated based on the wilt symptoms of seedlings. The wilt symptoms in the seedlings were observed in the morning on days 3, 7, 14, 21, 28, and 35 after the bacterial inoculation based on the scoring method as described in the previous study [7,17] with modifications. Score 0: all leaves or plants remained healthy with no symptoms; score 1: ≤20% withered leaves or plants; score 2: 21–40% withered leaves or plants; score 3: 41–60% withered leaves or plants; score 4: 61–80% of withered leaves or plants; and score 5: >80% withered leaves or plants/dead plant. Disease intensity (DI) and disease incidence (DI_{inc}), and the area under the disease progress curve (AUDPC) were calculated using the formula as referred to from the previous study [17,42,43], respectively. The DI was used to determine the resistance of each genotype, including the check cultivar. Genotypes were categorized as resistant if the DI was <30%, moderately resistant if the DI was 31–40%, moderately susceptible if the DI was 41–50%, and susceptible if the DI was >50% [17].

Experiment 2. Evaluation of relative gene expression JA and SA

The second experiment combined the responsiveness of the genotypes to mycorrhizae with their resistance to *R. solanacearum* to evaluate the relative gene expression of JA and SA. The treatments applied in this study consisted of seedlings that were not inoculated with mycorrhizae and not inoculated with *R. solanacearum* (M0R0), seedlings that were inoculated with mycorrhiza but not inoculated with *R. solanacearum* (M1R0), seedlings that were not inoculated with mycorrhiza but inoculated with *R. solanacearum* (M0R1),

and seedlings that were inoculated with mycorrhiza and inoculated with *R. solanacearum* (M1R1).

The seeds of the ten chili pepper genotypes were germinated in a germination box for 5 days. Twenty seedlings of each genotype were transplanted to nursery pot trays filled with sterilized growing media. The medium was a mixture of soil, coco peat, and husk charcoal at a ratio of 2:1:1 (*v:v:v*). Before transplanting the seedlings, the medium was mixed with the mycorrhizal inoculum at a ratio of 10:1 (*w:w*). The seedlings were maintained in the greenhouse until they were 21 days old.

The 21-day-old seedlings were transplanted into a 10-L planter bag filled with 7 kg of planting media per planter bag. The planting media was a mixture of soil, compost, coco peat, and husk charcoal at a ratio of 2:1:1:1 (*v:v:v:v*). The bacterium was inoculated on 21-day-old seedlings using the soil drenching method. A mixture of nine strains of the *R. solanacearum* suspension at a concentration of 1×10^8 CFU mL⁻¹ (OD600 = 0.1) was poured into the seedling media around the root area. Then, the seedlings were watered in the morning and afternoon, as required. A total of 20 planter bags were prepared for each genotype and repeated three times for each treatment. The bags were arranged in a completely randomized block design. The seedlings inoculated with *R. solanacearum* were placed in separate screen houses from those that were not inoculated. The plants were raised until they entered the generative growth phase, and leaf samples were taken (approximately 10 weeks after transplanting).

2.3. Isolation of Leaf RNA

The expression of JA and SA pathway genes was detected by quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) (BIO-RAD CFX96tm Real-Time System; Bio-Rad Laboratories, Hercules, CA, USA) using RNA samples extracted from leaf tissue. RNA was isolated from the youngest leaf tissue for qRT-PCR using the Geneaid RNA mini kit (Plant) [44]. A sample of 100 mg of leaf tissue was crushed in liquid nitrogen. The crushed tissue was placed in a 1.5 mL microtube and 500 µL of PRB Buffer and 5 µL of β-mercaptoethanol was added and mixed until homogeneous. The mixture was incubated at 60 °C for 5 min, transferred to a filter column with a 2 mL collection tube, and centrifuged at 3000 rpm (1000× *g*) for 1 min. Then, the filter column was discarded. The supernatant was taken from the collection tube and transferred to a 1.5 mL microtube. Absolute ethanol was added to the supernatant at half the volume of the total supernatant taken and homogenized with a vortex mixer. The supernatant was transferred to an RB column with a 2 mL collection tube and centrifuged at 12,000 rpm (16,000× *g*) for 1 min. The 2 mL of solution in the collection tube was discarded, the supernatant was returned to the RB column, and 400 µL of W1 buffer was added and centrifuged at 12,000 rpm (16,000× *g*) for 30 s. The solution in the collection tube was discarded and the supernatant was returned to the RB column. Then, 600 µL of wash buffer was added to the RB column, the eluate was centrifuged at 12,000 rpm (16,000× *g*) for 1 min, and the solution in the collection tube was discarded. The washing step with the wash buffer was repeated. The column tube was dried by centrifugation at 12,000 rpm (16,000× *g*) for 3 min. Then, the RB column was transferred to a 1.5 mL microtube, and 50 µL of RNase-free water was added to the center of the RB column and allowed to stand for 2 min. In the last stage, the eluate was centrifuged at 12,000 rpm (16,000× *g*) for 1 min, and the RNA was eluted into a 1.5 mL microtube.

2.4. qRT-PCR for GAPDH-*cp* and Genes of Interest

The qRT-PCR analysis was performed using the Bio-Rad CFX-96 real-time PCR system. The target genes were oxophytodienoic acid reductase-3 (OPR3) (*jasmonic acid*), and ICL (*salicylic acid*), and the reference gene was GAPDH-*cp* (Table 1). The total reaction volume was 20 µL, including 10 µL of 2 SensiFAST SYBR No-ROX One-Step Mix, 0.2 µL of reverse transcriptase, 0.4 µL of riboSafe RNase inhibitor, 1.6 µL of the primers (0.8 µL of the 10 µM forward primer and 0.8 µL of the 10 µM reverse primer), 4 µL of the RNA template, and

3.8 µL of RNase-free water. The qRT-PCR was implemented in three stages. Stage I was reverse transcription at 45 °C for 10 min, stage II was polymerase activation at 95 °C for 2 min, and stage III was 35 cycles of denaturation at 95 °C for 1 min, annealing for 30 s at the optimum temperature for each primer, elongation at 72 °C for 1 min, and a melting curve analysis at 65–95 °C for 5 s. The analysis was repeated in triplicate for each gene. The relative quantification of the genes of interest was assessed using the $2^{-\Delta\Delta CT}$ method [45].

Table 1. Primer and reference genes used in the real-time RT-PCR analysis.

Target	Primer ID	Sequence (5'–3')	Ta (°C)	References
Jasmonic acid	OPR3 Forward	CTTCTCATGCAGTGTATCAAC	46.4	[46]
	OPR3 Reverse	CGTCCAAGTGATCTATAGCTG		
Salicylic acid	ICL Forward	GCGTCGGAGGAAGTCAAGAA	50.5	[47]
	ICL Reverse	GGATTGGAAGCTGGAGCCGA		
Reference gene	GAPDH-Cp Forward	ATGATGATGTGAAAGCAGCG	46.4/50.5	-
	GAPDH-Cp Reverse	TTTCAACTGGTGGCTGCTAC		

2.5. Phenotypic Response of the Genotypes to Mycorrhizae and *R. solanacearum*

The phenotypic response was analyzed from the data of plant height and stem diameter of ten tropical chili pepper genotypes from all the treatments, i.e., M0R0, M1R0, M0R1 and M1R1. Plant height and stem diameter were measured at 10 weeks after transplanting. Plant height was measured from the ground surface to the shoot tip. The stem diameter was measured at approximately 5 cm above the ground. The data was collected from 15 plants for each genotype.

2.6. Data Analysis

The statistical analysis was performed using R Studio software version 3.6.2 [48]. The means of all observed traits were calculated using the least square means and analysis of variance. The Scott-Knott post hoc analysis was used to detect differences between the mean values of the observed characteristics. A p -value < 0.05 was considered significant. Structural equation modeling-partial least square (SEM-PLS) was used to construct the path coefficient and assess the cumulative effect of genotype responsiveness to mycorrhizae and the genotype resistance to *R. solanacearum* on the relative expression of the JA and SA genes [49,50]. A standardized stepwise regression was used to investigate the parameters of each genotype responsiveness variable to the mycorrhizae and the resistance of the chili genotypes to *R. solanacearum*, which affected the relative expression of the JA and SA genes [51]. SEM-PLS analysis and standardized stepwise regression were performed using PROC GLM and PROC REG in SAS 9.4 [52]. A heatmap was created using the Euclidean distance and the average heatmap method in R [53]. The heatmap was used to classify the genotypes based on their responsiveness to the mycorrhizae, resistance to *R. solanacearum*, and the relative gene expression of JA and SA. The heatmap was also used to determine the responsiveness to the mycorrhizae and/or the resistance to *R. solanacearum* related to the relative gene expression of JA and SA.

3. Results

3.1. Evaluation of the Growth Response to Arbuscular Mycorrhizae and Resistance to *R. solanacearum*

The growth response by the ten genotypes to the mycorrhizae was divided into three groups. Genotype C-38 had the highest growth response to the mycorrhizae, reaching $388.00\% \pm 37.40\%$, while genotype C-37 had the lowest growth response of $27.00\% \pm 2.60\%$. The growth response to mycorrhizae of four genotypes (C-08, C-30, C-09, C-38) was high; the responses of the other four genotypes (C-34, C-49, C-02, C-37) and the two commercial cultivars (C-50, C-54) were low (Figure 1).

All tropical chili pepper genotypes were infected with *R. solanacearum* 35 days after inoculation (DAI) but with different DI values. Among the eight accessions tested,

three were classified as resistant, one was moderately resistant, and four were susceptible genotypes (Figure 1). The susceptible check had a DI value of $67.19\% \pm 0.19\%$. The four accessions susceptible to *R. solanacearum* had DIs of 51.97–77.61%. The susceptible check in this evaluation resulted in DI, which was the same as the cultivar description. The resistant check cultivar had a DI of $16.22\% \pm 0.07\%$ at 35 DAI, which was the same as the cultivar description. Three accessions classified as resistant had higher DIs than the resistant check, ranging from 44.52 to 59.21% and 20.96 to 23.22%, respectively. One accession classified as moderately resistant had a DI of $31.91\% \pm 0.04\%$ (Figure 1).

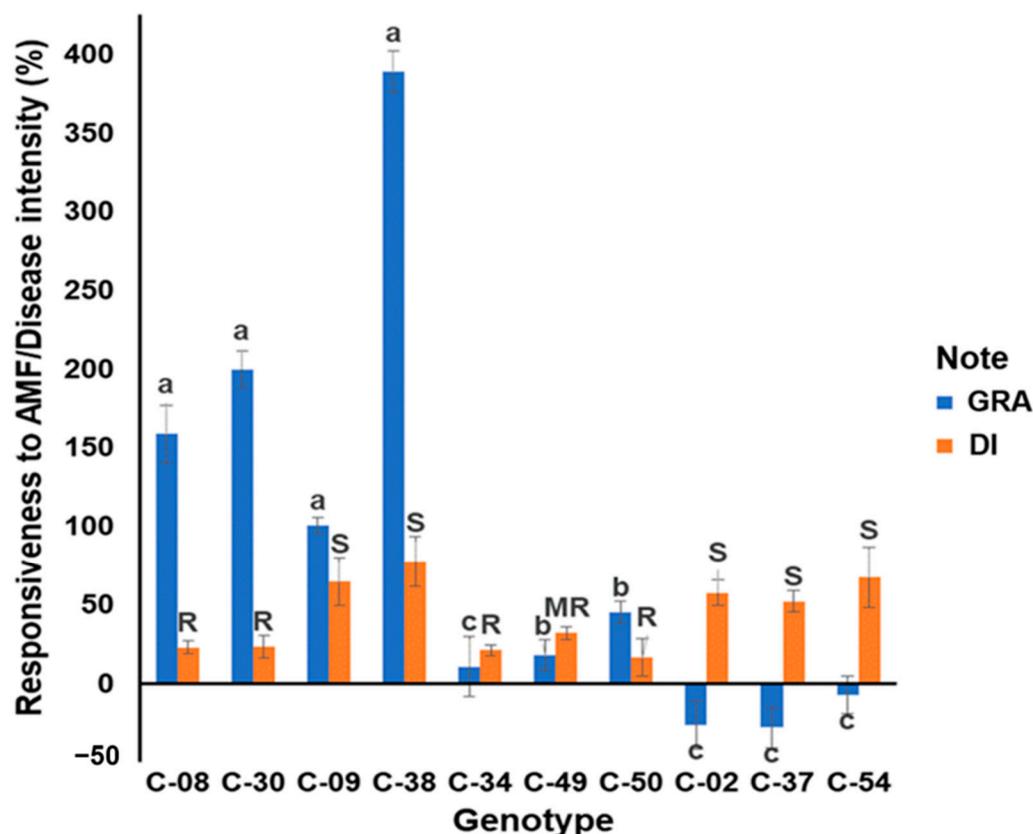


Figure 1. The growth responsiveness of the tropical chili pepper genotypes to mycorrhizae (GRA) and the disease intensity of the tropical chili pepper genotypes to *R. solanacearum* (DI) at 35 days after inoculation (DAI). Note: The same lowercase letters in the GRA histogram indicate no significant differences between the genotypes at a significance level of 0.95 according to the Scott-Knott test. The uppercase letters in the DI histogram indicate the resistance of the genotypes based on the disease intensity at 35 DAI. Bars represent the mean \pm standard deviation. AMF: arbuscular mycorrhizal fungi. GRA: the growth response of genotype to mycorrhiza; DI: disease intensity at 35 DAI. R: resistant; MR: moderately resistant; and S: susceptible; C-50: resistant check cultivar and C-54: susceptible check cultivar to *R. solanacearum* according to their published cultivar's description.

3.2. Effect of Arbuscular Mycorrhizae on the Relative Gene Expression of JA

The relative gene expression of JA in genotypes with high responsiveness to mycorrhizae and resistance to *R. solanacearum* (C-08 and C-30) was highest in the M1R1 treatment. In genotypes with high responsiveness to mycorrhizae and susceptibility to *R. solanacearum* (C-09 and C-38), the relative gene expression of JA was highest in M0R1. In genotypes with low responsiveness to mycorrhizae and susceptibility to *R. solanacearum* (C-02 and C-37), the relative gene expression of JA was highest in the M0R1 treatment. In genotypes with low responsiveness to mycorrhizae and resistance or moderate resistance to *R. solanacearum* (C-34 and C-49), the relative gene expression of JA was highest in M1R1 (Figure 2).

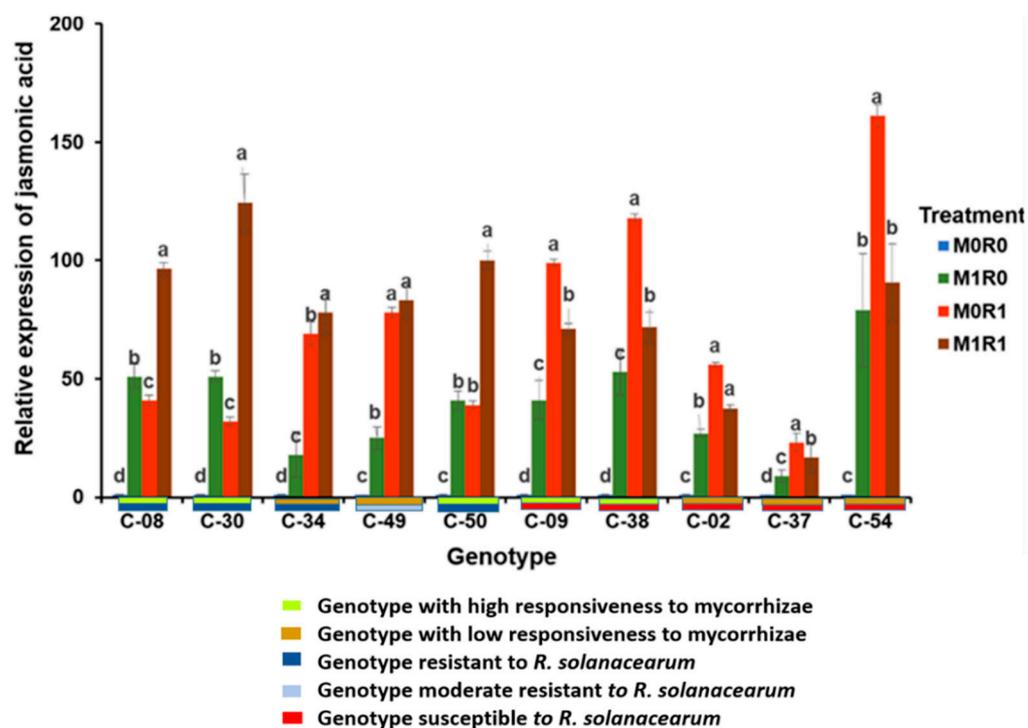


Figure 2. Relative gene expression of jasmonic acid (JA) in the tropical chili pepper genotypes. Note: Plants without mycorrhizae (M0); plants inoculated with mycorrhizae (M1); plants without *R. solanacearum* (R0); plants inoculated with *R. solanacearum* (R1). Bars represent the mean \pm standard deviation. The same lowercase letters indicate no significant difference in the mean values between treatments in each genotype, according to the Scott-Knott test, at a significance level of 0.95.

Inoculating mycorrhizae but not *R. solanacearum* (M1R0) showed that mycorrhizae caused the relative gene expression of JA to increase in the chili pepper genotypes. The relative gene expression of JA was higher in genotypes with high responsiveness to mycorrhizae than in those with low responsiveness. The relative gene expression of JA in the genotypes with high responsiveness to mycorrhizae ranged from 40.65 to 53.31 times, while the genotypes with low responsiveness only ranged from 9.33 to 27.20 times.

Inoculating with *R. solanacearum* but not the mycorrhizae (M0R1) showed that the relative gene expression of JA from the genotypes resistant to *R. solanacearum* was lower than that of the susceptible genotypes. However, the opposite result occurred in the genotypes resistant to *R. solanacearum*. The relative gene expression of JA from genotypes resistant to *R. solanacearum* ranged from 31.64 to 78.14. The relative gene expression of JA in the genotypes susceptible to *R. solanacearum* went from 38.38 to 118.04 times.

The genotypes inoculated with *R. solanacearum* and mycorrhizae (M1R1) triggered the relative gene expression of JA. The relative magnitude of JA gene expression depended on the resistance of the genotype to *R. solanacearum* and the responsiveness to the mycorrhizae. The relative gene expression of JA in the genotypes resistant to *R. solanacearum* increased in the range of 77.88–124.44 times. Genotypes susceptible to *R. solanacearum* in the presence of mycorrhizal symbiosis increased their relative gene expression of JA in the range of 16.88–71.71 times.

3.3. Effect of Arbuscular Mycorrhizae on the Relative Gene Expression of SA

The relative gene expression of SA in the genotypes with high responsiveness to mycorrhizae and resistance to *R. solanacearum* (M0R1) (C-08 and C-30) was the highest. The relative SA gene expression in the M1R1 and M1R0 treatments was higher than in the M0R1 in the resistant check cultivar (C-50). In the genotypes with high responsiveness to mycorrhizae and susceptibility to *R. solanacearum* (C-09 and C-38), the relative gene

expression of SA in the M1R1 treatment was highest. The relative gene expression of SA in the resistant check cultivar was highest in the M1R1 treatment. In the genotypes with low responsiveness to mycorrhizae and susceptibility to *R. solanacearum* (C-02 and C-37), the relative gene expression of SA was highest in the M1R1 treatment. In the genotypes with low responsiveness to mycorrhizae and resistance or moderate resistance to *R. solanacearum* (C-34 and C-49), the relative gene expression of SA was highest in the M1R1 treatment, followed by M1R0 and M0R1 (Figure 3).

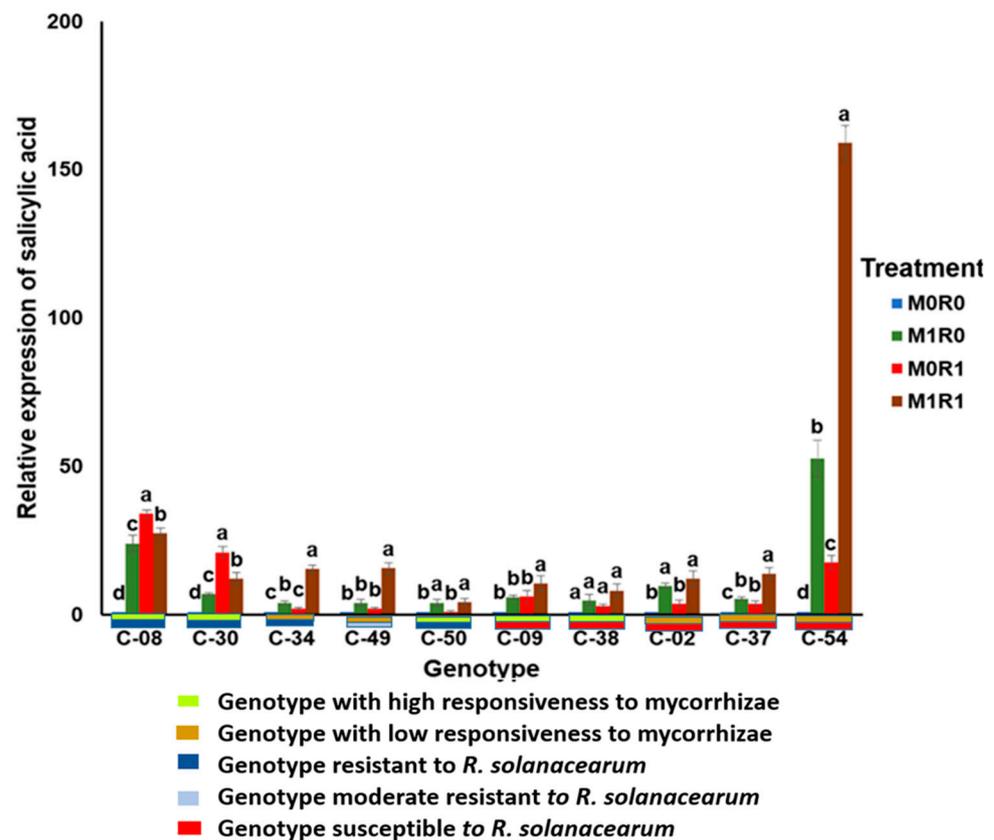


Figure 3. Relative gene expression of salicylic acid (SA) in the tropical chili pepper genotypes. Note: Plants without mycorrhizae (M0); plants inoculated with mycorrhizae (M1); plants not inoculated with *R. solanacearum* (R0); plants inoculated with *R. solanacearum* (R1). Bars represent mean \pm standard deviation. The same lowercase letters indicate no significant difference in mean values between treatments in each genotype according to the Scott-Knott test at a significance level of 0.95.

Genotypes inoculated with mycorrhizae but not with *R. solanacearum* (M1R0) showed the relative gene expression of SA depended on the responsiveness of the genotype to the arbuscular mycorrhizal. Genotypes with high responsiveness to the mycorrhizae revealed higher relative gene expression of SA than genotypes with low responsiveness. The range of relative gene expression of SA in the responsive genotypes was 4.81–23.7 times, which was higher than those in genotypes with low responsiveness at 4.15–9.63 times.

The resistant genotypes tended to express more SA than the susceptible genotypes. This observation can be seen in the genotypes treated with bacterial inoculation without mycorrhizae (M0R1). The relative gene expression range of SA in the resistant genotypes was 2.93–34.21 times, which was higher than that in the susceptible genotypes at 2.02–17.68 times.

The combined inoculation treatment with *R. solanacearum* and mycorrhizae (M1R1) showed that the arbuscular mycorrhizal symbiosis increased the relative gene expression of SA. The increase in relative SA expression depended on the resistance of the genotype to *R. solanacearum*. In the presence of mycorrhizae, the relative gene expression of SA in the

resistant genotypes ranged from 12.16 to 74.45 times. It tended to be higher than that in the susceptible genotypes which were only 8.13–13.81 times.

3.4. Structural Equation Modeling-PLS and Standardized Stepwise Regression

The results of the SEM-PLS analysis showed that the resistance to *R. solanacearum* had a significant path coefficient on the relative gene expression of JA ($p < 0.048^*$) (Figure 4). However, the relative gene expression of SA was not directly affected by either the responsiveness to mycorrhizae or the resistance to *R. solanacearum* (Figure 5).

The standardized stepwise regression showed that the relative gene expression of JA was significantly affected by the percentage colonization of mycorrhizae (PCA) (0.935^{***}) ($R^2 = 0.874^{***}$) and the DInc (0.9480^{***}) ($R^2 = 0.928^{***}$). Similarly, the relative expression of the SA gene was significantly affected only by DInc (0.648^*) ($R^2 = 0.648^*$). Thus, the PCA is a responsiveness variable, and DInc is a variable of resistance. Therefore, the relative gene expression of JA was strongly affected by the responsiveness of the tropical chili pepper genotypes to mycorrhizae and the resistance to *R. solanacearum*. The relative gene expression of SA was related to the resistance of the tropical chili pepper genotypes to *R. solanacearum*.

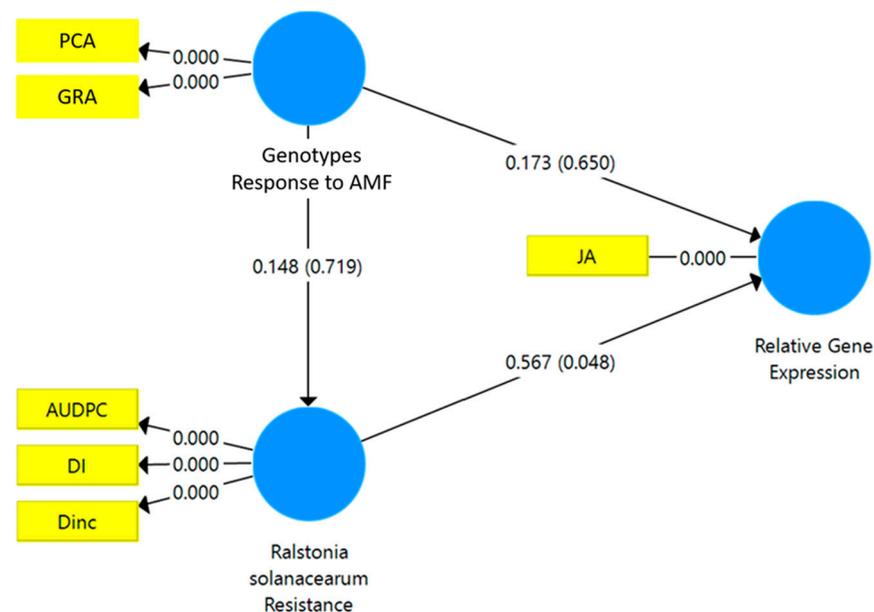


Figure 4. Structural equation modeling-partial least square (SEM-PLS) of the relationship between the responsiveness to mycorrhizae and the resistance of the tropical chili pepper genotypes to the *R. solanacearum* variables and the relative gene expression of JA. AMF: arbuscular mycorrhizal fungi. PCA: percentage colonization of mycorrhiza, GRA: the growth response of the tropical chili pepper genotype to mycorrhizae, DI: disease intensity, DInc.: disease incidence, AUDPC: area under disease progress curve, JA: jasmonic acid.

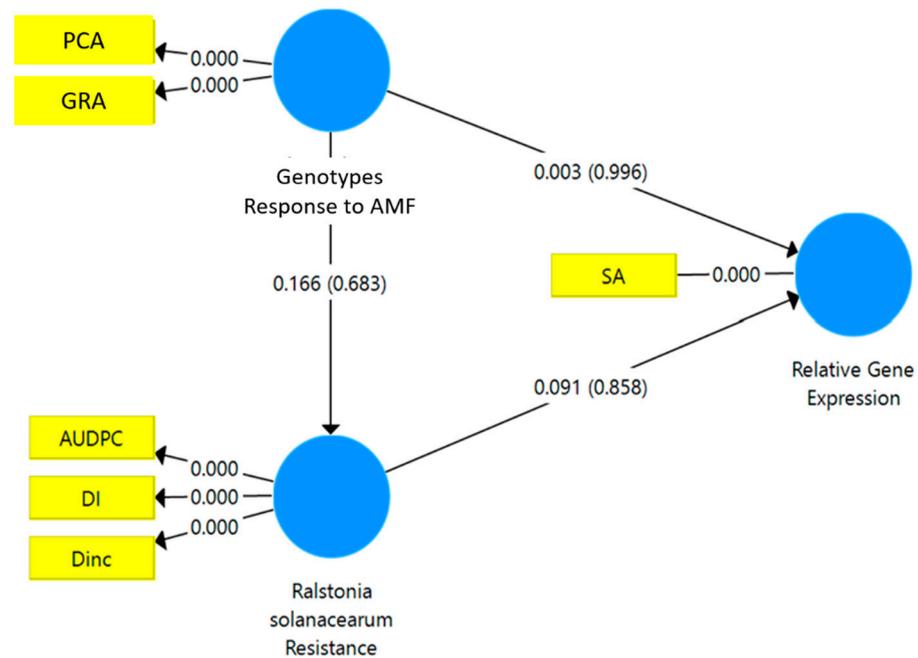


Figure 5. Structural equation modeling-partial least square (SEM-PLS) of the relationship between the responsiveness to mycorrhizae and the resistance of the tropical chili pepper genotypes to *R. solanacearum* variables and the relative gene expression of SA. AMF: arbuscular mycorrhizal fungi. PCA: percentage colonization of mycorrhizae, GRA: the growth response of the tropical chili pepper genotypes to mycorrhizae, DI: disease intensity, DInc.: disease incidence, AUDPC: area under disease progress curve, SA: salicylic acid.

3.5. Clustering of the Genotypes Based on the Responsiveness to Mycorrhizae, Resistance to *R. solanacearum*, and the Relative Gene Expression of JA and SA

The genotypes were grouped into four clusters based on the heatmap (Figure 6). The heatmap shows high diversity in several traits, i.e., PCA, genotype responsiveness to mycorrhizae (GRA), DI, DInc, the AUDPC, and the relative gene expression of JA and SA. The relative variation in JA and SA gene expression in the tropical chili pepper genotypes was similar (Figures 2 and 3). The heatmap shows that the relative gene expression of SA was related to variable resistance of the genotypes to *R. solanacearum*. In contrast, the relative gene expression of JA was related to variable resistance of the genotypes to *R. solanacearum* and the GRA. The results of this heatmap agree with the standardized stepwise regression results regarding the relative determinants of JA and SA gene expression.

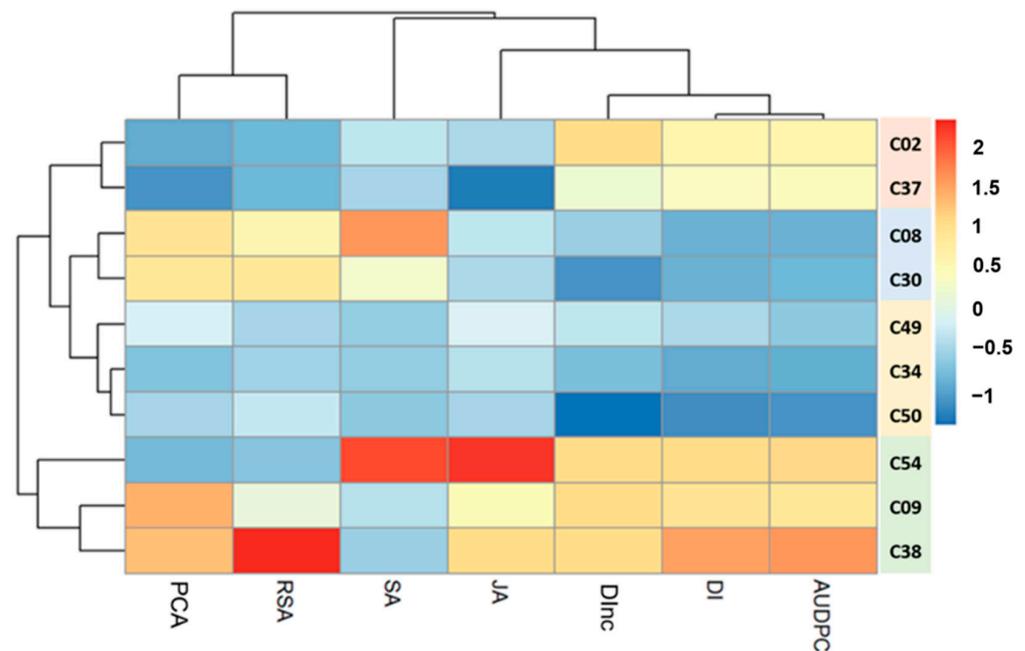


Figure 6. Heatmap describing the diversity of responsiveness to the mycorrhizae, the resistance to *R. solanacearum*, and the relative gene expression of JA and SA. Blue and red indicate an increase and decrease in the value of each trait, respectively. The black lines on the top and at the left of the heatmap indicate dendrogram of variables and genotypes, respectively. Color-bar on the right side of the dendrogram indicates the genotypes in the same cluster. PCA: percentage colonization of mycorrhiza, GRA: genotype responsiveness to mycorrhiza, SA: salicylic acid, JA: jasmonic acid, DInc: disease incidence, DI: disease intensity, AUDPC: area under the disease progress curve.

3.6. Phenotypic Response Based on the Responsiveness to Mycorrhizae and Resistance to *R. solanacearum*

Figure 7 shows the response of ten genotypes of tropical chili pepper based on responsiveness to mycorrhizae, resistance to *R. solanacearum*, and relative gene expression of JA and SA on plant height. In general, the genotypes inoculated with *R. solanacearum* and arbuscular mycorrhizae (M1R1) were able to suppress the growth of *R. solanacearum*, the plants were taller than plants infected *R. solanacearum* but not inoculation mycorrhizae (M0R1). The same phenomenon occurs in the variable stem diameters of ten genotypes with various mycorrhizal responsiveness and *R. solanacearum* resistance, mycorrhizae were able to suppress the growth of *R. solanacearum* (Figure 8). The relative gene expression of JA and SA induced by mycorrhizae in the presence of *R. solanacearum* (M0R1) could suppress the growth of *R. solanacearum*. This was indicated by the higher plants and larger stem diameters of the tropical chili pepper genotypes than the plants infected *R. solanacearum* but not inoculation mycorrhizae (M0R1) (Figures 7 and 8).

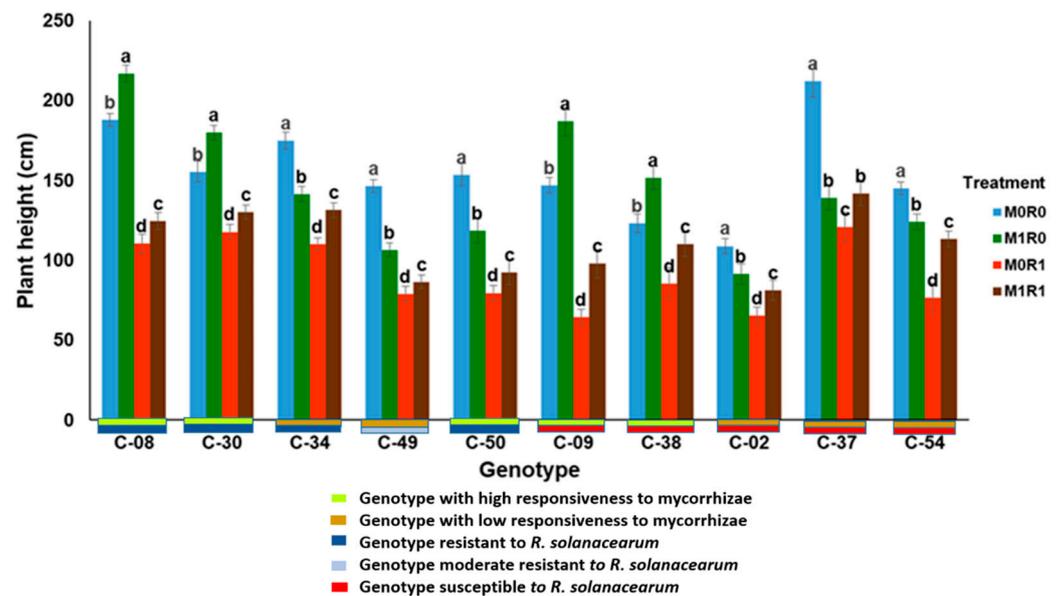


Figure 7. Plant height of ten genotypes tropical chili pepper as a phenotypic response to the treatments. Bars represent mean \pm standard deviation. The same lowercase letters indicate no significant difference in mean values between treatments in each genotype according to the Scott-Knott test at a significance level of 0.95.

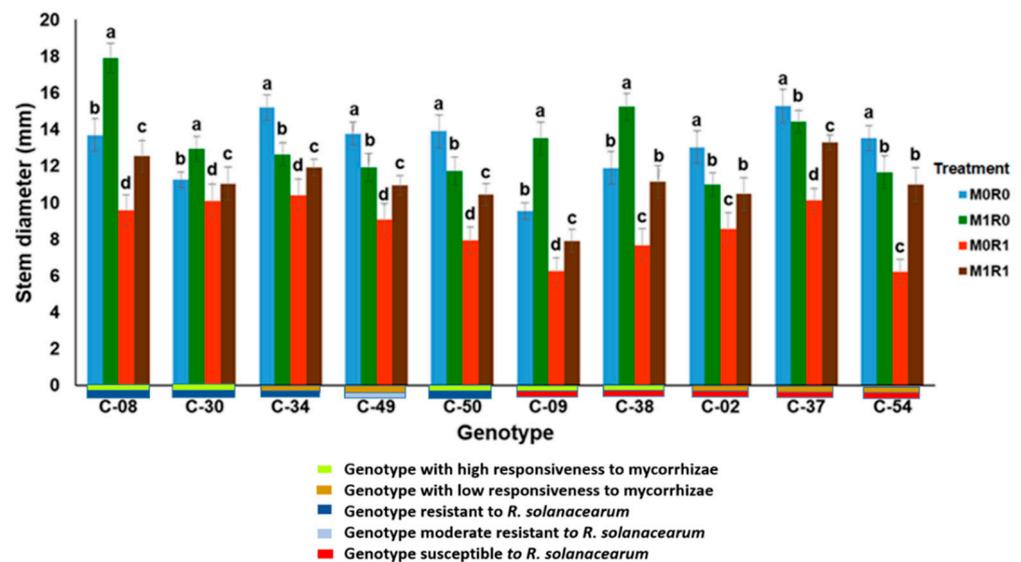


Figure 8. Stem diameter of ten genotypes tropical chili pepper as a phenotypic response to the treatments. Bars represent mean \pm standard deviation. The same lowercase letters indicate no significant difference in mean values between treatments in each genotype according to the Scott-Knott test at a significance level of 0.95.

4. Discussion

4.1. Responsiveness to Arbuscular Mycorrhizae and the Resistance to *R. solanacearum*

Figure 1 shows the diverse growth responses of the tropical chili pepper genotypes to the mycorrhizae. The genotypes differed in their response to arbuscular mycorrhizal colonization. The effectiveness of the symbiosis with mycorrhizae is highly dependent on the species and the genotype of the host plant [54,55]. The genotype is an essential factor determining the response to mycorrhizae [31,56,57]. Differences in the response to mycorrhizae emphasize a genetic basis for the interactions between plants and mycorrhizae [31]. Many studies have reported significant differences in the responsiveness to mycorrhizae between

genotypes within the same host plant species [27,54,57]. The symbiosis with arbuscular mycorrhizae changed the growth of the tropical chili peppers. Janos [58] stated that the responses of plant genotypes to mycorrhizal colonization can be classified into positive, neutral, and negative. The positive reactions of four of the tropical chili pepper genotypes indicated that mycorrhizal colonies and the host plant got benefit from the symbiosis. A small positive symbiotic response indicated that the tropical chili pepper as the host plant did not respond to the presence of mycorrhizae, and the mycorrhizae did not interfere with the host plant. This observation indicates that there was no effect on the host plant in the presence of arbuscular mycorrhizal symbioses.

In contrast, a negative response was found in two of the tropical chili pepper genotypes, as the mycorrhizal symbioses inhibited plant growth or induced symbiotic parasitism. The reaction to the mycorrhizae is neutral or negative in cases where mycorrhizal colonization does not increase plant growth [27]; the biomass produced by plants does not increase even though the plants are well colonized with mycorrhizae [57].

Screening for resistance to *R. solanacearum* in chilies has been carried out but has focused on red chilies and sweet pepper (*Capsicum annuum*). Several sources of resistance to bacterial wilt disease have been identified in *Capsicum* pepper [12,42,59–64]. Three chili pepper genotypes from the germplasm were resistant, one was moderately resistant, and four were susceptible (Figure 1). These results demonstrate that resistant genotypes can be obtained from Indonesia, as reported by several researchers [62,64,65]. This also explains why Southeast Asia is an essential source of chili germplasm resistant to *R. solanacearum* bacterial wilt disease. The germplasms were more adapted to the various environment. They collected represented centers of chili pepper production areas in the three provinces in Jawa Island. Resistant genotypes are used as a source of resistance genes in breeding programs. As *R. solanacearum* easily adapts to the environment, including infecting resistant host plants, the screening results in this study could be used to breed new resistant cultivars.

4.2. Interaction between the Genotypes of Tropical Chili Pepper, the Arbuscular Mycorrhizae, and *R. solanacearum* on the Relative Gene Expression of JA and SA, and Phenotypic Response

Arbuscular mycorrhizal fungi change the community and the biotic interactions in the rhizosphere. This mycorrhizospheric effect alters the communities of beneficial microorganisms and suppresses pathogens in the soil [66–68]. Associations with mycorrhizae directly benefit plants by producing secondary metabolites or modulating plant defenses. A recent study showed that different endophytes induce plant resistance to biotic or abiotic stressors [32,69]. However, the plant microbiome is strongly influenced by its host. Plants must recognize endophytes during colonization, as signaling molecules, and identifying microbes as beneficial partners will create a symbiotic mutualism.

All of these factors affect different plant species in different microbiomes [19]. This study investigated the function of the symbiosis of chili pepper and mycorrhizae and the resistance to *R. solanacearum*. Ten tropical chili pepper genotypes with different responsiveness to mycorrhizae and resistance to *R. solanacearum* were tested. The function of the mycorrhizae was evaluated and the relative gene expression of JA and SA in each tropical chili pepper genotype was determined.

JA is an essential hormone involved in the plant resistance system. JA regulates various processes related to plant development, symbiotic interactions, and plant responses to pathogens, particularly soil-borne pathogens. JA triggers ISR, which is activated by the relationship between the plant and certain beneficial microorganisms. ISR is marked by the accumulation of JA [19,32,33]. The relative gene expression of JA in the ten tropical chili pepper genotypes is shown in Figures 2 and 6. In all ten tropical chili pepper genotypes, treatment with mycorrhizae and *R. solanacearum* infection (M1R1) increased the relative gene expression of JA. The increase in the relative gene expression of JA varied (Figure 2). For the resistant and moderately resistant genotypes (with blue bar under the X-axis), the relative gene expression of JA was higher in the M1R1 treatments. In this genotype group, the difference between M1R1 and M0R1 was larger in the genotypes which are responsive

to mycorrhizae (with green and blue bar under the X-axis). It means that the effect of mycorrhizae was higher in the genotypes with high responsiveness to mycorrhizae. In contrast, the relative gene expression of JA in the five susceptible genotypes was higher in the MOR1 treatment (orange histograms with red bar under the X-axis). This finding indicates that JA is always expressed in the infected plants, but with different expressions between resistant and susceptible plants. For resistant plants, the relative expression of JA was depended on responsiveness to mycorrhiza. Genotypes C-08, C-30, and C-50 were resistant to *R. solanacearum* and had a high responsiveness to mycorrhizae. It showed a high expression of JA even in the treatment without infection. Meanwhile, for the susceptible plants, the expression of JA depended mainly on the presence *R. solanacearum* infection.

R. solanacearum infection increased the expression of the OPR3 gene, and the relative gene expression of JA was higher (Figure 2). The SEM-PLS in Figure 4 shows a relationship between JA gene expression and the resistance of the genotypes to *R. solanacearum*. The heatmap revealed similar results (Figure 6). As the accumulation JA was altered by inoculating the endophytic fungi, it can be argued that the expression of several marker genes for different steps in the JA biosynthetic pathway and other genes is associated with plant resistance [19].

The M1R0 treatment increased the relative gene expression of JA in all ten tropical chili pepper genotypes. However, differences were observed in the relative gene expression of JA. The relative gene expression of JA was higher in genotypes with a high mycorrhizal response than in those inoculated with *R. solanacearum* only (MOR1). A similar result was found for the resistant check cultivar. However, this result differed from genotypes with high responsiveness to the mycorrhizae but susceptibility to *R. solanacearum* in which the relative gene expression of JA was the lowest. This result is the same as in genotypes classified as poor responders to arbuscular mycorrhizae. A similar result was found in the susceptible check cultivar. The JA signaling involved in symbiosis must be modulated to maintain arbuscular mycorrhizal fungal endophytes in the asymptomatic stage [19,38].

The relative gene expression of JA in the ten genotypes inoculated with arbuscular mycorrhizae and *R. solanacearum* (M1R1) increased, even in the genotypes with a low or negative response to the mycorrhizae. The mycorrhizae increased the relative gene expression of JA in all ten tropical chili pepper genotypes that were inoculated with *R. solanacearum*. Based on the standardized stepwise regression, the relative gene expression of JA was significantly affected by the DInc and the PCA. The heatmap analysis provided the same results (Figure 6).

In general, antagonism occurs between the JA and SA biosynthetic pathways. Thus, if the relative gene expression of SA is high, the relative gene expression of JA is low. These hormonal signaling pathways do not act independently but affect each other through a complex network of regulatory interactions [35]. Several researchers have reported synergistic interactions between the JA and SA biosynthetic pathways [36,37]. Arbuscular mycorrhizal symbioses increased the production of SA. The increase was not dependent on the genotype's responsiveness to mycorrhizae, but it depended on their resistance to *R. solanacearum*. The results of the standardized stepwise regression reinforced this result. The relative gene expression of SA was significantly affected by DInc., either the mycorrhizal inoculation only (M1R0) or both arbuscular mycorrhizal and *R. solanacearum* (M1R1) inoculation.

It is known that the presence of endophytic mycorrhiza can mediate plant resistance by changing endogenous hormones. Symbiosis with endophytic fungi is usually associated with inhibiting the SA pathway and increasing JA production [19,38]. The results showed a significant increase in the relative gene expression of the JA and SA pathways, indicating that mycorrhizal colonization of the tropical chili pepper genotypes increased plant defense. Similarly, the tropical chili pepper genotypes inoculated with mycorrhizae increased their relative gene expression of JA, and the increase was higher than that of SA (Figures 2 and 3). The expression of JA and SA were in the opposite way. When JA was expressed higher, the SA would be low, and vice versa. Mycorrhizal colonization induced gene expression,

activated the JA pathway, and increased plant resistance to *R. solanacearum*. JA levels increased significantly, particularly in the genotypes inoculated with mycorrhizae and infected with *R. solanacearum* (M1R1). Figures 7 and 8 showed a comparison of plant height and stem diameter of chili pepper plants with and without mycorrhiza inoculation. It may represent an increase in plant resistance to *R. solanacearum*. The chili pepper plant inoculated with mycorrhiza and *R. solanacearum* (M1R1) showed a higher plant height and thicker stem diameter compared to the chili pepper plant inoculated with *R. solanacearum* but without mycorrhiza (M0R1). Mycorrhizal colonization protected the tropical chili pepper from *R. solanacearum* and enhanced the immune priming system by producing JA and SA in genotypes with high and low responsiveness or hostile responses to mycorrhizae. However, the increase in the relative gene expression of the JA and SA signaling pathways was affected by the resistance properties of the tropical chili pepper genotypes to *R. solanacearum*.

As biotrophic organisms, mycorrhiza can trigger plant defense responses during the early stages of colonization, similar to biotrophic pathogen infection [67,70]. Plant responses occur in defense of colonization. Modulating plant defense responses leads to tissue preconditioning to activate plant defenses systemically during a pathogen attack by activating JA signaling, which is referred to as priming. Plant responses are faster and more robust in dealing with a pathogen infection when primed so they are more efficient at increasing plant resistance and plant health [66,67,71,72]. This priming phenomenon was found in the mycorrhizae and *R. solanacearum* (M1R1) inoculated treatment, especially in the genotypes resistant to *R. solanacearum*. The relative gene expression of JA increased in the five genotypes of tropical chili peppers in the M1R1 treatment, regardless of the responsiveness of the chili pepper to the mycorrhizae (Figure 2).

Resistance to *R. solanacearum* is closely related to and controlled by JA and SA-mediated signaling [73]. Mycorrhizae potentially control plant diseases, particularly soil-borne pathogens [26–28]. The presence of mycorrhizae induces the reprogramming of plant metabolic pathways involved in defense, which may increase the ability of the plants to survive adverse conditions [5,19,23–25]. Local and systemic MIR increases the content of plant hormones, such as SA and JA [23,24,29–31].

Symbiosis with mycorrhizae increases the root surface area more than 100 times compared to non-symbiotic roots [74]. Colonization with arbuscular mycorrhizae changes the root morphology, and these changes make it difficult for soil-borne pathogens to enter the root system. The symbiosis directly causes an increase in mycorrhizal exudation from root branches, which changes the root microbiome and stimulates root exudates that inhibit pathogens. This also increases the acquisition of mineral nutrients and modulates plant hormonal balance. Arbuscular mycelia protect the roots from the damaging effects of various pathogenic hydrolytic enzymes that disrupt the epidermal cell walls. Arbuscular mycelia block the molecular crosstalk required for the host plant/pathogen interaction with the rhizosphere [23,24,75].

5. Conclusions

Among the eight accessions, three groups were resistant to *R. solanacearum*. Three genotypes were resistant, one genotype was moderately resistant, and four genotypes were susceptible to *R. solanacearum*. Based on the responsiveness to the arbuscular mycorrhizae, the ten genotypes were classified as four tropical chili pepper genotypes with high responsiveness and six with low responsiveness to the arbuscular mycorrhizae, three of which exhibited negative responsiveness. Inoculating the tropical chili peppers with arbuscular mycorrhizae induced resistance to *R. solanacearum* through a priming action by increasing the relative expression of the genes associated with JA and SA. However, the relationship was different between the JA and SA pathways. Mycorrhizae induced the relative expression of the gene associated with JA in the tropical chili peppers, which depended on the responsiveness to mycorrhizae and resistance to *R. solanacearum*. The relative expression of the gene associated with SA was depended on the resistance of the genotypes to *R. solanacearum*. These results suggest that mycorrhizal colonization modulates the signaling

pathways mediated by JA and SA to activate plant defense genes against bacterial wilt caused by *R. solanacearum* and increase plant resistance.

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