BIODIVERSITAS
Volume 23, Number 10, October 2022
Pages: 5314-5322

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d231041

Expression of *SIX1b* and *SIX1c* effector genes and banana resistance genes during *Foc* TR4 infection on banana cultivars

AZIZAH RIDHA ULILALBAB[1], SRI WIDINUGRAHENI[3], MASANTO[4], SITI SUBANDIYAH[1,2], ARIF WIBOWO[1,♥]

[1]Department of Plant Pests and Diseases, Faculty of Agriculture, Universitas Gadjah Mada. Jl. Flora 1, Bulaksumur, Sleman 55281, Yogyakarta, Indonesia. Tel./fax.: +62-274-563062, ♥email: arif_wibowo@ugm.ac.id

[2]Research Center for Biotechnology, Universitas Gadjah Mada. Jl. Teknika Utara, Depok, Sleman 55281, Yogyakarta, Indonesia

[3]Department of Agrotechnology, Faculty of Agriculture, Universitas Nusa Cendana. Jl. Adi Sucipto Penfui No. 1, Lasiana, Kupang 85001, East Nusa Tenggara, Indonesia

[4]Tanjung Priok Agricultural Quarantine Service, Indonesian Agricultural Quarantine Agency. Jl. Enggano No. 17, Tanjung Priok, North Jakarta 14310, Jakarta, Indonesia

Manuscript received: 9 June 2022. Revision accepted: 15 October 2022.

Abstract. *Ulilalbab AR, Widinugraheni S, Masanto, Subandiyah S, Wibowo A. 2022. Expression of* SIX1b *and* SIX1c *effector genes and banana resistance genes during* Foc *TR4 infection on banana cultivars. Biodiversitas 23: 5314-5322.* The relative expression *SIX1* genes isolates of *Foc* TR4 and banana resistance genes have not been intensively studied. This study aimed to determine the expression of *SIX1* genes of *Foc* TR4 and banana resistance genes. Twelve *Foc* TR4 isolates were collected from several regions in Indonesia. DSI analysis was performed to categorize their virulence levels. The representative *Foc* TR4 isolates from different virulence levels were artificially inoculated on the *Musa acuminata* Colla Cavendish subgroup and cv. Barangan and incubated for 48, 72, and 96 HPI. The expressed genes were quantitatively analyzed using the qPCR technique using primers to amplify *SIX1b*, *SIX1c*, chitinase, and PR-protein 1 genes. The results categorized the virulence of *Foc* TR4 isolates into moderate, virulent, and high virulent. The isolates of KJG and Batu-4 were selected for gene expression study representing moderate and high virulent groups, respectively. The results of in planta assay found that the expression of *SIX1b*, *SIX1c*, chitinase, and PR-protein1 was upregulated on the inoculated plants during the incubation period. However, the expression of these genes was increasingly upregulated in both bananas at early-stage inoculation. We assumed that plant defense genes of bananas might actively encounter the common virulence mechanisms of *Foc* TR4 at the initial stage of inoculation.

Keywords: Chitinase, *Foc* TR4, gene expression, *Musa acuminata, SIX1*

INTRODUCTION

Banana (*Musa* spp.) is one of the important crops in the world and a source of income in many developing countries. Approximately 5.6 million ha of land is dedicated to banana production globally, and it represents the fifth most agricultural crop in world trade in 2017 (FAO 2022). Plant diseases constrain banana production worldwide and in Indonesia, including *Fusarium* wilt (Wibowo et al. 2011; Yanti et al. 2018). The disease causes a highly destructive impact economically, moreover, the yearly losses of bananas in the world are 60-90% (Bhuvanendra et al. 2010). *Fusarium oxysporum* f.sp. *cubense* (*Foc*) is highly variable. There are four races of *Foc* have been identified (Aoki et al. 2014). Race 4 has a broad host range infecting almost all cultivars, including Cavendish (*Musa* sp. AAA group) as well as the hosts of races 1 and race 2 (Guo et al. 2014). Tropical race 4 (*Foc* TR4) is highly virulent on almost all banana cultivars and destroying global commercial banana Cavendish subgroup worldwide among *Foc* races (Magdama et al. 2019)

A comprehensive and integrated disease management strategy is highly required to minimize the economic loss due to this disease. One of the common diagnostic targets is the gene-encoding proteins strongly correlated with the pathogen virulence (Carvalhais et al. 2019). In *F. oxysporum*-infected tomato caused by *Fusarium oxysporum* f.sp. *lycopersici*/*Fol*, some of these proteins have been detected in the xylem sap and named *Secreted in Xylem (SIX)* (Maldonado et al. 2018). In *Fol*, this effector is required during the early stages of infection. Its expression is induced during root penetration and continues through invasion of xylem vessels but is not detected upon plant cell death (van Der Does 2008). Interestingly, three copies of the *SIX1 gene*, namely *SIX1a*, *SIX1b*, and *SIX1c* are present in the genome of FocTR4 (Guo et al. 2014). According to Widinugraheni et al. (2018), *Foc SIX1 gene* of *Foc* TR4 was required for full virulence of *Foc*TR4 in Cavendish bananas as an important virulence factor. However, there is no information regarding its temporal expression.

Fifty isolates of *Foc* have previously been collected from several banana production regions in Indonesia representing various groups and molecularly confirmed as *Foc* using FocEf3 primer sets. Twelve isolates were confirmed as *Foc* TR4 using some primer sets in the previous study (Pratama et al. 2018). They were KD-2, Ciamis, Batu-2, KJG, Batu-3, Prembun, Sdt-1a, BDG-1a, GnK2, Pujon, KP-3, and Batu-4. A hundred plant defense-

related genes have been classified, one of them is on banana defense response against *Fusarium* (Swarupa et al. 2014). Pathogenesis-related proteins have been divided into 17 families of induced proteins. PR1 is the dominant group induced by pathogens or salicylic acid, and is usually used as a marker of pathogen-induced Systemic Acquired Resistance (SAR), conferring on plants the enhanced defensive state (Ali et al. 2018). The PR1 transcript level has been increasingly found in each genotype in response to the *Foc* TR4 attack (Li et al. 2015). Several proteins coded by DEGs identified in Pahang may play key roles in the banana-*Foc* TR4 interaction, including pathogen-related protein 1 and chitinase (Zhang et al. 2019).

Plants and pathogens have formed complex relationships of competitive interactions. Therefore, less genomic knowledge of *Foc* is a problem in arranging preventive control management. This study will provide basic information about early critical time interaction between host plant defense and pathogen virulence genes. Therefore, this study aimed to determine the expression of *SIX1* genes and banana defense genes of those isolates under artificial inoculation on banana plants. Our interest study characterized and evaluated the gene expression of some *SIX genes* and defense genes at early infection events during *Foc*TR4-banana.

MATERIALS AND METHODS

Research materials

The research samples were 12 *Foc*TR4 isolates from the culture collection of the Department of Entomology and Plant Pathology, Universitas Gadjah Mada (UGM), Indonesia (Table 1). They were previously collected from several banana production regions in Indonesia representing various groups of VCG, races, genotype, and banana cultivars, which had been molecularly confirmed as *Foc* TR4 using the specific primer sets described by Pratama et al. (2018).

Procedures

Preparation of Foc isolates

Twelve *Foc*TR4 isolates were cultured on a PDA medium and incubated for 7 days. The spore suspension was prepared by adding sterile water to the plate culture and adjusting the spore density at approximately 107 cfu/mL using a haemacytometer (Jayatri et al. 2018).

In vitro virulence test using banana (Musa acuminata Cavendish subgroup)

The experiment was performed with 13 artificial *Foc* TR4 inoculation treatments, and five plant replicates. *Musa acuminata* Colla Cavendish subgroup seedlings with 5 mature leaves were collected from Banana Tissue Culture laboratory at the Center for Horticultural Seedlings Institute in Magelang, Central Java, Indonesia. They were individually transplanted in a plastic pot containing a sterile soil medium before inoculation. Artificial inoculation was implemented by wounding the surrounding roots and pouring the volume of 25 mL of microconidia suspension at 10^7 microconidia/mL density into the rhizosphere. The seedlings were maintained for two months before inoculation.

The genome of selected isolates was checked using Polymerase Chain Reaction (PCR) with seven primers (Table 2). Firstly, the selected isolates were cultured in Potato Dextrose Agar (PDA) medium in petridish and incubated for 1 week at room temperature. Then, DNA was extracted using Genomic DNA Mini Kit (Plant) Protocol Geneaid. Then, polymerase Chain Reaction (PCR) amplifications were performed in a total volume of 50 µL containing 19 µL of miliQ water, 25 µL of PCR mix (RedMix), 2 µL of primer forward, 2 µL of primer reverse, and 2 µL of DNA extract of *Fusarium oxysporum* f.sp. *cubense*. PCR amplifications were carried out using the PeqStar XS PCR machine. Programs set as follows: the first step was initial denaturation in one cycle using 95°C for 1 minute. The next step was denaturation using 95°C for 15 seconds, annealing based on user-determined for 15 seconds, and extension using 72°C for 10 seconds. Finally, all of them were repeated in 35 cycles.

In planta experiment of Foc *TR4 inoculation on* M. acuminata *Cavendish subgroup and* M. acuminata *cv.* Barangan *plantlets*

The plantlets of banana cultivars Cavendish and Barangan were used in this experiment for the moderate resistant and susceptible cultivars, respectively, against *Foc* TR4. These uniform plantlets (with 3-4 leaves) were grown in Murashige and Skoog medium. The *Foc* isolates with different virulence levels (moderate and high virulent) were cultured on PDA and incubated at room temperature for a week. Their roots were wounded by a scalpel, soaked into spore suspension, and then incubated for 48, 72, and 96 h. Wounded roots in plantlets without inoculating the pathogen were considered a negative control.

RNA extraction and cDNA synthesis from control and inoculated model plant

The total mRNA of the inoculated and uninoculated plantlets at 48, 72, and 96 HPI were extracted using Total RNA Mini Kit (*GENE*aid Biotech Ltd., New Taipei City, Taiwan).

Table 1. Origin of isolates of *Foc* from previous study

Isolates of *Foc*	Origin
KD-2	Yogyakarta
Pujon-5	East Java
Prembun	Central Java
Ciamis-1	West Java
Sdt-1a	Central Java
KP-3	Yogyakarta
Batu 2	East Java
BDG1-a	West Java
Batu 4	East Java
GnK 2	Yogyakarta
KJG-2	Kalimantan
Batu 3	East Java

Table 2. List of primers used in the study

No.	Primer name	Sequence (5'-3')	Product length	References
1	FocEf3_F	CGTTCTGGCACGATTATTCAC	600	Pratama et al. (2018)
	FocEf3_R	GGGGGGGAGAATTAACGAAC		
2	FocSIX1b-F	GGGAGTGTCCCAGATAACAGTG	92	Poon et al. (2019)
	FocSIX1b-R	CGTCTCGGTCTGAACACTATCG		
3	FocSIX1c-F	CCAGAGGGGCAGGCTCAG	96	Poon et al. (2019)
	FocSIX1c-R	GTAGACTTGTCCGTGGTAGGCGAC		
4	β-tubulin-F	CCCCGAGGACTTACGATGTC	62	Sutherland et al. (2013)
	β-tubulin-R	CGCTTGAAGAGCTCCTGGAT		
5	PR-protein-F	ACAAGTTCGAGTGCAAGCAG	122	Design primer for this study
	PR-protein-R	CACAACCTTCAACACACATCCC		
6	Chitin-F	GCTGCAGGCAAGAAATACTACG	131	Design primer for this study
	Chitin-R	ACGCAGAACACATCGGATTG		
7	Musa-Actin-F	TGTTGCATCCTGGTACTGCT	112	Liu et al. (2020)
	Musa-Actin-R	GGCTTTCTTGCACTGGTACAC		

Amplification using qPCR

A total of 20 µL of PCR reaction contained 3.8 µL ddH_2O, 10 µL 2× SensiFAST™ SYBR® No-ROX One-Step Kit (Bioline) PCR ready mix, 0.8 µL forward primers, 0.8 µL reverse primers, 0.2 µL reverse transcriptase, 0.4 µL ribosafe RNAse inhibitor, and 4 µL RNA template. The mixture was run under condition as follows: reverse transcriptase at 45ºC for 10 min; polymerase activation at 95ºC for 2 min; 40 cycles of denaturation at 95ºC for 5 s, annealing at 55ºC for 10 s and extension at 72ºC for 5 s; and final extension at 72ºC for 5 min. Meanwhile, the melt profile was adjusted to the instrument of Bio-Rad CFX96 (Bio-Rad). The primers used in the study are shown in Table 2.

Data analysis

The virulence level of *Foc* was determined by measuring the Disease Severity Index (DSI) based on Leaf Symptom Index (LSI) and rhizome discoloration observed weekly for 11 weeks after inoculation (Kiswanti et al. 2010) (Tables 3, 4, and 5).

DSI was then calculated based on LSI and RDI data using the following formulation:

$$\text{DSI} = \frac{\sum(score\ x\ number\ of\ corresponding\ plants)}{\sum(number\ of\ tested\ plants)}$$

The virulence level of *Foc* isolates was determined according to LSI and RDI data (Table 5). Two representative samples with different virulence levels were selected for the in planta qPCR analysis.

The analysis of relative gene expression data was conducted using the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen 2001). The expression of virulence-related genes was quantitatively compared with the housekeeping gene. We used actin and β-tubulin for housekeeping genes of plant defense genes of banana and virulence-related genes of *Foc*, respectively. Their relative expression was illustrated in graph Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) and heat map comparison using ClustVis web tool (Metsalu and Vilo 2015).

Table 3. Leaf Symptoms Index (LSI)

Scoring	Remark
0	No symptoms / healthy plant
1	1-2 chlorosis / wilting leaves
2	3-4 chlorosis / wilting leaves
3	5 chlorosis / wilting leaves
4	>5 chlorosis / wilting leaves

Table 4. Rhizome Discoloration Index (RDI)

Scoring	Remark
0	No discoloration/rotting on rhizome and rooting area or surrounding tissues
1	No discoloration/rotting on rooting area, discoloration found on root branches only
2	Discoloration rotting up to 5% on rhizome
3	Discoloration rotting up to 6-20% on rhizome
4	Discoloration rotting up to 21-50% on rhizome
5	Discoloration rotting up to >50% on rhizome
6	Rotting on whole parts of rhizome and rooting area
7	Plant died

Table 5. Remarks on DSI Scale

Scale of DSI for LSI	Scale of DSI for RDI	Remarks
0	0	Avirulent
0.1-1	0-2.0	Moderate
1.1-2	2.1-4	Virulent
2.1-3	4.1-7	High virulent

RESULTS AND DISCUSSION

Virulence level test on seedling of *Musa acuminata* Cavendish subgroup

The level of disease severity could be observed from external symptoms, such as yellowing and wilting leaves and internal symptoms, such as rotting of rhizomes. The result showed that the ranges of DSI for LSI and RDI were 0-2 and 0.8-4.8, respectively. These data categorized five *Foc* TR4 isolates into moderate virulent (KD-2, Ciamis-1, Batu-2, KJG, and Batu-3), four *Foc* TR4 isolates were virulent (Prembun, Sdt-1a, BDG-1a, and GnK-2) and other three *Foc* TR4 isolates were grouped into high virulent (Pujon-5, KP-3, and Batu-4) (Table 6). The isolates of KJG and Batu-4 were then selected for in planta experiment and qPCR analysis representing moderate and high virulent isolates, respectively. All of the isolates were positive to specific primer sets of *Fusarium oxysporum* f.sp. *cubense* tropical race 4 by Pratama et al. (2018).

Disease symptom was observed on the 4th-6th weeks after inoculation, whereas the maximum disease incidence and severity was documented on the 6th and 11th weeks after inoculation, respectively (Figures 1 and 2). Diseases incidence of *Fusarium* wilt in banana showed that all the treated banana were 100% infected by *Foc* on the 9th after inoculation (Figure 1). Diseases severity of *Fusarium* wilt in banana were 100% infected by all samples *Foc* in the last observation on 11th weeks after inoculation. It was started on the 5th weeks after inoculation with values under 20%, and increased until on the 11th week. It showed that inoculation of treated banana was successfully infected by *Foc* (Figure 2).

The symptoms showed yellowing, wilting leaves, and rotting of rhizomes on the treated banana. The wilting seedlings with rhizome discoloration were recorded on treated banana, while healthy seedlings with clear rhizome were observed on the untreated banana (Figure 3).

The PCR result of selected isolates was shown that *SIX1c* is present on both KJG and Batu-4. Meanwhile, only on KJG isolate that *SIX1b* is present, whereas in Batu-4 *SIX1b gene* is absent (Figure 4). The disease symptoms emerged on the 4th-6th weeks after inoculation of both KJG and Batu4 treatments.

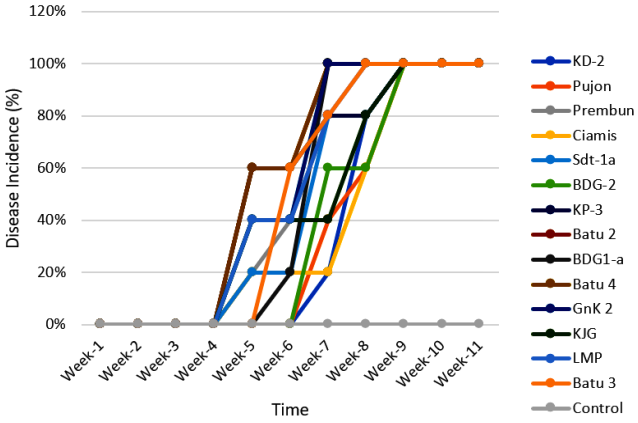

Figure 1. Diseases incidence of *Fusarium* wilt in *M. acuminata* Cavendish subgroup

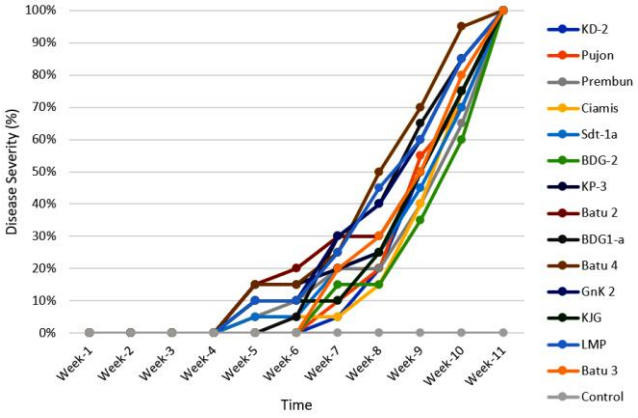

Figure 2. Diseases severity of *Fusarium* wilt in *M. acuminata* Cavendish subgroup

Table 6. Virulence test of isolates of *Fusarium oxysporum* f.sp. *cubense* TR4 in *M. acuminata* Cavendish subgroup

Isolates of *Foc*	DSI based on LSI	DSI based on RDI	Remarks of DSI based LSI and RDI	*Foc* TR4
KD-2	0.8	2	Moderate	+
Pujon-5	0.8	4.8	High Virulent	+
Prembun	0.6	4	Virulent	+
Ciamis-1	0.6	2	Moderate	+
Sdt-1a	0.8	3.6	Virulent	+
KP-3	1	4.2	High Virulent	+
Batu-2	1.2	1	Moderate	+
BDG-1a	1.6	4	Virulent	+
Batu-4	2	4.2	High Virulent	+
GnK-2	1.6	3	Virulent	+
KJG	1	1.2	Moderate	+
Batu-3	1.2	0.8	Moderate	+

Note: +: as *Foc* TR4, -: Negative toward specific primer sets of *Foc* TR4

Figure 3. *Musa acuminata* Cavendish subgroup after inoculation with isolate Batu 4 and KJG of *Fusarium oxysporum* f.sp. *cubense* (*Foc*) TR4 (Original scale 1:15)

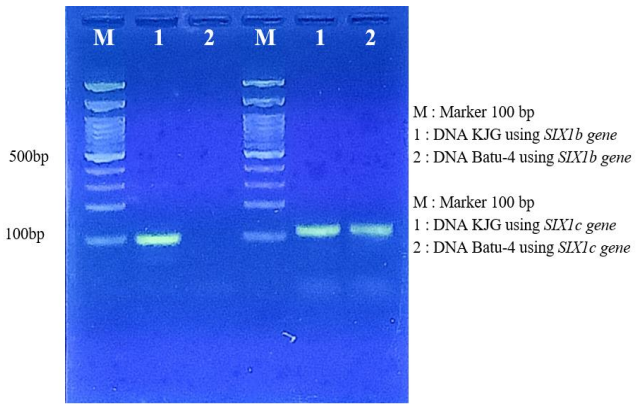

Figure 4. PCR visualization of DNA genome of isolate Batu-4 and KJG *Fusarium oxysporum* f.sp. *cubense* (*Foc*) TR4 using *SIX1b* and *SIX1c genes*

The expression of virulence-related genes and plant defense using qPCR analysis

The selected isolates to be tested for gene expression was positively detected with PCR using *SIX1b* and *SIX1c* primer sets. The DNA of the highest virulent isolate (Batu-4) was absent, it was found not to be amplified using the *SIX1b* primers. It was caused the highest virulent isolate was absent of *SIX1b gene*. The expression of *SIX1b* and *SIX1c genes* were tested and documented at 48, 72, and 96 h after inoculation (Figures 5 and 6). The gene of *SIX1b* was highly expressed only from moderate *Foc* TR4 isolate (KJG) and not to the Batu-4 isolate as the highest virulence isolate. Whereas *SIX1b* was less expressed on high virulent isolate. *SIX1c gene* was upregulated both on moderate and high virulent *Foc* TR4 isolates. Both the high virulent and moderate isolates were applied to infect two different banana seedlings as susceptible (Barangan) and resistant (Cavendish) hosts. Interestingly, higher expression of *SIX1b* gene is shown at Cavendish KJG than Barangan KJG (Figure 7). It suggests that these genes were fluctuatively expressed in infecting both susceptible and moderate resistant banana cultivars or could this gene play a role as an avirulent gene.

Analysis of qPCR revealed that plant defense-related *GENE*s on these two banana cultivars were induced due to the infection of both moderate and high virulent isolates of *Foc* TR4. They were highly upregulated at 48, 72, and 96 Hours Post Inoculation (HPI) (Figures 8 and 9). However, the expression of *PR-protein 1* was higher than that of chitinase on both susceptible and moderate resistant banana seedlings and maximum expression was found at 72 HPI. They were generally more expressed in moderate resistant banana *M. acuminata* Cavendish subgroup rather than on the susceptible banana *M. acuminata* cv. Barangan (Figure 10).

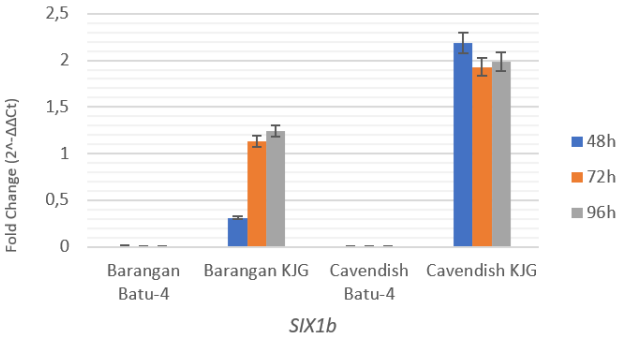

Figure 5. Relative expression of *SIX1b gene* of cDNA *Fusarium oxysporum* f.sp. *cubense* TR4 at 48, 72, and 96 HPI (hours post inoculation). Two different isolates were tested, Batu-4 and KJG, which are indicated as highly virulent and as moderate isolates, respectively

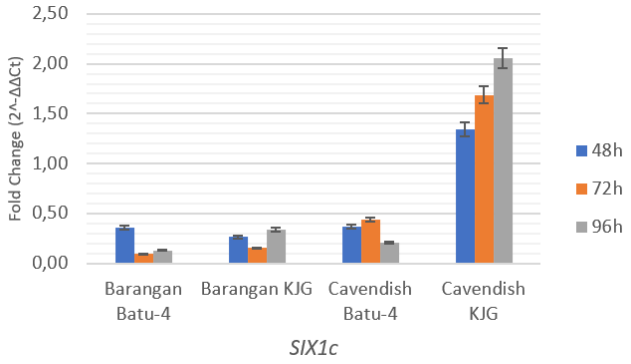

Figure 6. Relative expression of *SIX1c gene* of cDNA *Fusarium oxysporum* f.sp *cubense* TR4 at 48, 72, and 96 HPI. Two different isolates were tested, Batu-4 and KJG, that are indicated as highly virulent and as moderate isolates, respectively

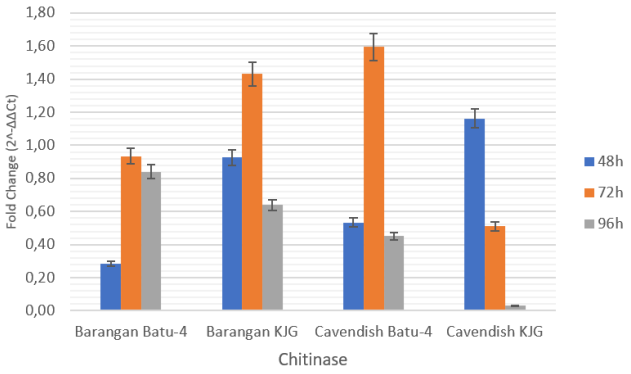

Figure 9. Relative expression of *chitinase GENE* in *M. acuminata* Cavendish subgroup and *M. acuminata* cv. Barangan; Batu-4 set as high virulent and KJG as moderate isolates

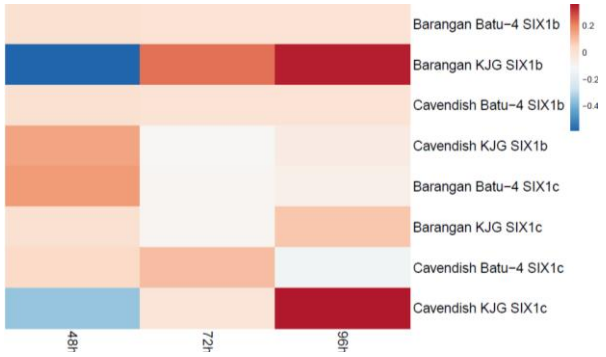

Figure 7. Heatmap comparison of expressed *SIX GENE*s in moderate and high virulent isolates of *Fusarium oxysporum* f.sp. *cubense* TR4 in *M. acuminata* Cavendish subgroup and *M. acuminata* cv. Barangan

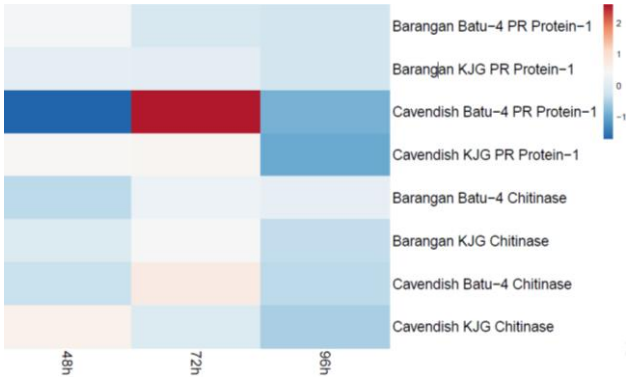

Figure 10. Heatmap comparison of expressed *PR-Protein1* and *Chitinase GENE*s in moderate and high virulent isolates of *Fusarium oxysporum* f.sp. *cubense* TR4 in *M. acuminata* Cavendish subgroup and *M. acuminata* cv. Barangan

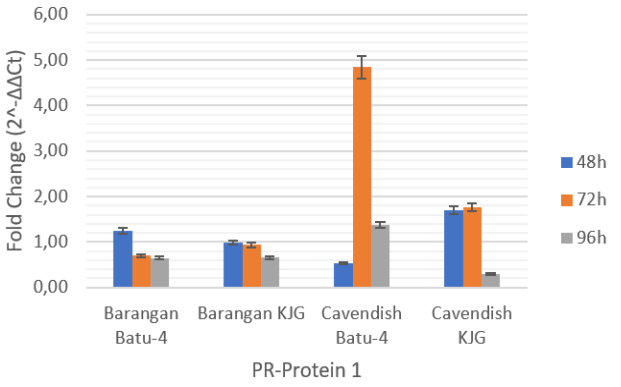

Figure 8. Relative expression of *PR-Protein1 GENE* in *M. acuminata* Cavendish subgroup and *M. acuminata* cv. Barangan; Batu-4 set as high virulent and KJG as moderate iso

Discussion

This study might be considered the first study on the co-expression of potential plant defense-related genes of banana from different resistance levels and virulence-related genes of *Foc* from various virulence levels, particularly *Foc* TR4 in Indonesia. Previously, Widinugraheni et al. (2018) reported a *SIX1* homolog contributing to the virulence of *Foc* TR4 without observing the expression of plant defense-related genes in banana. This study would also evaluate the specificity of specific primer sets developed for *Foc* TR4 (VCG 01213) originating from different banana production areas in Indonesia (Pratama et al. 2018).

The range of appearance of initial symptoms indicated the variation in aggressiveness among the tested *Foc* TR4 isolates. Dita et al. (2014) noted that the incubation period of *Foc* on banana might be variable depending on the inoculation procedures, banana genotypes used, and aggressiveness of the *Foc* isolates or the environmental condition. We wounded the plantlet to enable the pathogen to penetrate and be introduced into plant tissue to grant the success of the inoculation process. The successful infection process would generate the symptom in the host plant

(Gabrekiristos et al. 2022). Further investigation with an inoculation procedure without any wounding is required to ensure the natural penetration capability of this pathogen on the host plant. The disease incidence showed 100% infection of *Fusarium* wilt in the *M. acuminata* Cavendish subgroup, which means that the artificial inoculation was a success. The symptoms showed yellowing, wilting leaves, and rotting of rhizomes on the treated banana. The wilting seedlings with rhizome discoloration were recorded on the treated banana, while healthy seedlings with clear rhizomes were observed on the untreated banana (Figure 3).

Three different virulence levels of these 12 *Foc* TR4 isolates might be due to the difference in biological, chemical, genetic, and ability of asexual spore reproduction on each isolate (Groenewald 2005). Therefore, the dominant moderate virulent isolates might illustrate the common virulence level of *Foc* TR4 on moderate-resistant banana cultivars. However, the use of a moderate resistant banana cultivar for the assay of the virulence level of *Foc* in this study could be biased due to the resistance level of the host plant. Therefore, it is suggested to use a susceptible cultivar as the host plant in an artificial inoculation test of virulence level for uniformity.

A virulence level test on the *M. acuminata* Cavendish subgroup seedlings was used for choosing the selected isolates of *Foc* for the next step. The selected isolates were KJG as moderate isolates and Batu-4 as highly virulent. The selected isolates of KJG (moderate virulent) and Batu-4 (high virulent) were not compatible with VCG 01213, and they confirmed *Foc* TR4 using some primers sets (Wibowo et al. 2011; Pratama et al. 2018) (Table 6). According to Pratama et al. (2018), the virulence level of those *Foc* TR4 isolates was generally independent of their compatibility group, origin host plant, and geographical areas.

The tested virulence-related genes, *SIX1b* and *SIX1c* were part of *SIX1* genes which had been found in *Foc*TR4 (Guo et al. 2014). *SIX1a* has been recently characterized by Widinugraheni et al. (2018) through a knockout approach, providing evidence of an important virulence factor in *Foc* TR4. Previously, the involvement of these effectors was detected in the pathogenesis of *Fol* from the xylem sap of infected-tomato plants (Rep et al. 2004; Gawehns et al. 2015). There are 14 *SIX gene*s (*SIX1* to *SIX14*) encoding small secreted proteins that have been identified, most of which are cysteine-rich (Rep et al. 2004). Further study is required to determine the potential of other *SIX1 gene*s in the pathogenesis mechanism of *Foc* TR4. It has been reported that knocking out *SIX1* in *Foc* TR4 severely reduced the virulence of the *M. acuminata* Cavendish subgroup (Widinugraheni et al. 2018). However, the functions of other *SIX gene*s remain unknown. According to Ahmad et al. (2020), virulence level did not correlate with the existence or lack of these genes. Virulence genes may be involved in the association between genetic variability and *Foc* aggressiveness (Sutherland et al. 2013; Ellis et al. 2016).

The upregulated expression of *SIX1 gene*s in this research agreed with van der Does et al. (2008) that the strongly upregulated expression of *SIX1 gene*s in *Fol* is due to the presence of the host. Their early expression at 48 HPI complied with the recent report of Poon et al. (2019), suggesting the induction of the expression of *SIX1b* and *SIX1c* at the initial stage of infection in the host cells. Our study recorded the clear expression of *SIX1c gene* in both moderate and high virulent *Foc* TR4 isolates inoculated on either susceptible or moderate resistant banana cultivars, suggesting the prevalence of this gene as one of the virulence-related genes in *Foc* TR4. Meanwhile, the unexpressed *SIX1b gene* in highly virulent isolate probably indicated its low prevalence or absence in such virulence level pathogen. It was also supported by the results of PCR amplification revealing the absence of DNA genome bands of moderate virulent isolates under the detection using *SIX1b* primer sets (Figure 4).

Two banana cultivar was susceptible to *Foc* TR4. Cultivar Barangan was highly susceptible to *Foc* TR4, whereas Cavendish was moderately susceptible. Higher expression was shown in the combination between *M. acuminata* Cavendish subgroup than cv. Barangan in *SIX1b* and *SIX1c*. It revealed that KJG and Batu-4 needed more effort to infect the *M. acuminata* Cavendish subgroup than cv. Barangan, so the level expression of *SIX1b* and *SIX1c* was highly expressed in Cavendish. The level expression of *SIX1b* was shown that the combination between KJG-*M. acuminata* Cavendish subgroup was high than KJG-*M. acuminata* cv. Barangan, meanwhile, Batu-4 was absent in *SIX1b gene*. That the results showed KJG as a moderate isolate through virulence assay needed more effort to infect banana, especially Cavendish, so the expression level of *SIX1b* was high in combination with KJG-*M. acuminata* Cavendish subgroup. It was a similar reason to *SIX1c gene*. Based on (Figure 6) the dominant expression level of *SIX1c* gene was shown at KJG-*M. acuminata* Cavendish subgroup, the reason was KJG as a moderate isolate through virulence's assay needed more effort to infect banana, especially the *M. acuminata* Cavendish subgroup. According to Wang et al. (2020), only Six1, Six6, and Six9 were identified among the SPs of *Foc* R1 or *Foc* TR4. The Six1 homolog (Six1) was identified in *Foc* R1, while the Six1 homologs proteins (Six1a, Six1b, Six1c) were not identified in *Foc* TR4. This finding seems to be inconsistent with the absolute requirement for Six1a for full toxicity of *Foc* TR4 (Widinugraheni et al. 2018), while it is also possible that Six1 does not play a role in the early stage of invasion by *Foc* TR4.

Analysis of qPCR revealed that plant defense-related genes on these two banana cultivars were induced due to the infection of both moderate and high virulent isolates of *Foc*. They were highly upregulated at 48, 72, and 96 HPI (Figures 8 and 9). However, the expression of *PR-protein 1* was higher than that of chitinase on both susceptible and moderate-resistant banana seedlings, and maximum expression was found at 72 HPI (Figure 10). They were generally more expressed on the moderate resistant banana cultivar of Cavendish rather than the susceptible banana cultivar of Barangan (Figure 10). Therefore, it can be assumed that in Cavendish subgroup is higher containing *PR-protein1* than cv. Barangan. For chitinase

genes, both moderate resistant and susceptible resistance of bananas was the same.

The higher expression of PR-protein 1 in this experiment was parallel to previous research (Subramaniam et al. 2006; Li et al. 2015), proving it as one prominent feature of plant defense responding to biotic stress. It was one of the cell wall degrading enzymes in addition to PR3 and PR10, digesting the major components of chitin or glucan in fungi cell walls for inhibiting fungal growth. Its maximum expression at 72 HPI complied with the prior findings of Li et al. (2015). These findings could provide valuable information for molecular plant-microbe interaction.

The expression of chitinase from this current study was similar to Zhang et al. (2019), suggesting the possible involvement of chitinase in response to *Foc* TR4. It was an enzyme induced by the plant hormone ethylene or pathogen attack, inhibiting fungal growth in vitro and accumulating around fungal hyphae in vivo by degrading chitin as an important component of fungal cell walls (Malik and Preety 2019; Vaghela et al. 2022). First, however, it is important to find out the low appearance of this enzyme by tracing the whole genome sequences of susceptible and moderate resistant banana cultivars in the next experiment.

Our findings proved the presence of plant defense-related genes in both susceptible and resistant banana cultivars as well as their expression due to the induction of either moderate or high virulent *Foc* TR4 isolates. These could be potential genes for developing resistant banana cultivars against *Foc* TR4. Rocha et al. (2022) showed that the chitinase gene was more highly expressed in the BRS Platina cultivar than in the Prata-Anã and Grand Naine cultivars at 12 hours after inoculation, which may be closely related to the resistance of this cultivar in the early stage of infection of *Foc*. It showed that the prevalence of chitinase was a potential gene for resistance genes. According to Zhang et al. (2019) PR1 maintained up-regulation in both banana genotypes during *Foc* TR4 infection. In line with these studies, it can be a potential gene for resistance genes. However, the prevalence of other similar genes should be explored more and a very early expression prior to 48 HPI is needed to recognize to figure out a comprehensive defense mechanism for those genes in banana, particularly in responding *Foc* TR4 infection. More expression of plant defense-related genes on resistant banana cultivars revealed a great potential for optimizing the resistant plant materials in controlling *Fusarium* wilt disease on banana. Such information may be studied, primarily their genomic or proteomic aspects.

In conclusion, *Foc* TR4 isolates were categorized into three virulence levels, i.e., virulent, moderate, and high virulent, based on yellowing wilt leaves and rhizome discoloration symptoms. The potential plant defense-regulated genes, chitinase and PR-protein 1, were expressed as the response to the induction of both moderate and high virulent *Foc* TR4 isolates, in which the higher expression was documented on moderate resistant banana cultivars. Virulence-related genes of *SIX1b* and *SIX1c* generally remained upregulated in both moderate and high virulent *Foc* TR4 isolates, infecting different resistance levels of banana cultivars with the higher expression in high virulent *Foc* TR4 isolates. For both *SIX1b* and *SIX1c*, higher expression was shown in combination between *M. acuminata* Cavendish subgroup rather than that on the susceptible banana *M. acuminata* cv. Barangan. So, the level expression of *SIX1b* and *SIX1c* was highly expressed in Cavendish. It suggests that these genes were fluctuatively expressed in infecting susceptible and moderate resistant banana cultivars, or could this gene play a role as an avirulent gene. It must be continued the study about the function of *SIX1b* and *SIX1*c *GENES* that plays a role in virulence genes is still unknown. This insight may contribute to understanding the plant-microbe interaction in the case of Fusarium wilt disease on bananas for developing resistant planting materials.

ACKNOWLEDGEMENTS

We thank ACIAR fund agricultural research in the East Asian Island Nation of Indonesia and *Perhimpunan Fitopatologi Indonesia* (PFI) for being financially supported. We also thank Nur Fathurahman Ridwan and Nanang Setyawan for their assistance in RNA extraction and qPCR preparation.

REFERENCES

Ahmad S, Wong CKF, Vadamalai G, Wahab MA, Saidi NB, Zulperi D. 2020. Molecular characterization of *Fusarium oxysporum* f.sp *cubense* tropical race 4 (*Foc*-TR4) isolates from Malaysia banana using *secreted in xylem* (SIX) effector genes. Arch Phytopathol Plant Prot 53 (1): 1-17. DOI: 10.1080/03235408.2020.1761766.

Ali S, Ganaib BA, Kamilib AN, Bhatc AA, Mira ZA, Bhatd JA, Tyagia A, Islame ST, Mushtaqd M, Yadava P, Rawata S, Grovera A. 2018. Pathogenesis-related proteins and peptides as promising tools for engineering plants with multiple stress tolerance. Microbiol Res 212-213: 29-37. DOI: 10.1016/j.micres.2018.04.008.

Aoki T, O'Donnell K, Geiser DM. 2014. Systematics of key phytopathogenic *Fusarium* species: Current status and future challenges. J Gen Plant Pathol 80: 189-201. DOI: 10.1007/s10327-014-0509-3.

Bhuvanendra HK, Udaya Shankar AC, Chandra NS, Ramachandra KK, Shetty HS, Prakash HS. 2010. Biochemical characterization of *Fusarium oxysporum* f.sp. *cubense* isolates from India. Afr J Biotechnol 9 (4): 523-530. DOI: 10.5897/AJB09.1069.

Carvalhais LC, Henderson J, Rincon-Florez VA, O'Dwyer C, Czislowski E, Aitken EAB, Drenth A. 2019. Molecular diagnostic of banana *Fusarium* wilt targeting secreted in xylem genes. Front Plant Sci 10: 1-17. DOI : 10.3389/fpls.2019.00547.

Dita MA, Vicente LP, Martinez E. 2014. Inoculation of *Fusarium oxysporum* f.sp *cubense* causal agent of *Fusarium* wilt in banana. Memorias de XV Reunion Internacional de ACORBAT (Oaxaca, MX).

Ellis ML, Lanubile A, Garcia C, Munkvold GP. 2016. Association of putative fungal effectors in *Fusarium oxysporum* wilt symptoms in soybean. Phytopathology 106 (7): 762-773. DOI: 10.1094/PHYTO-11-15-0293-R.

FAO. 2022. Food and Agriculture Statistical Databases (FAOSTAT). https://www.fao.org/faostat. accessed June 2022.

Gabrekiristos E, Yesuf M, Ayana G. 2022. An optimized inoculation method of *Fusarium* wilt (*Fusarium oxysporum* f.sp. *cubense*) causal agent of banana wilt disease in Ethiopia. Greener J Plant Breed Crop Sci 6 (2): 7-14. DOI: 10.15580/GJPBCS.2018.2.048918065.

Gawehns F, Ma L, Bruning O, Petra M. Houterman PM, Boeren S, Cornelissen BJC, Rep M, Takken FLW. 2015. The effector repertoire of *Fusarium oxysporum* determines the tomato xylem proteome

composition following infection**.** Front Plant Sci 6: 1-17. DOI: 10.3389/fpls.2015.00967.

Groenewald S. 2005. Biology, Pathogenecity, and Diversity of *Fusarium oxysporum* f.sp. *cubense*. [Dissertation]. University of Pretoria, South Africa.

Guo L, Han L, Yang L, Zeng H, Fan D, Zhu, F, Feng Y, Wang G, Peng C, Jiang X, Zhou D, Ni P, Liang C, Liu L, Wang J, Mao C, Fang X, Peng M, Huang J. 2014. Genome and transcriptome analysis of the fungal pathogen *Fusarium oxysporum* f.sp. *cubense* causing banana vascular wilt disease. PLoS ONE 9: 1-17. DOI: 10.1371/journal.pone.0095543.

Jayatri HN, Sumardiyono C, Wibowo A. 2018. Race and virulence determination of *Fusarium oxysporum* f.sp *cubense* isolates from Sidomulyo Village of Bantul, Yogyakarta. Jurnal Perlindungan Tanaman Indonesia 22: 72-81. DOI: 10.22146/jpti.26283. [Indonesian]

Kiswanti D, Suryanti, Sumardiyono C. 2010. Identification and virulence of *Fusarium oxysporum* f.sp *cubense* race 4. Jurnal Perlindungan Tanaman Indonesia 16 (1): 28-32. [Indonesian]

Li W, Ge X, Wu W, Wang W, Hu Y, Mo Y, Sun D, Shi S, Xie J. 2015. Identification of defense-related *GENE*s in banana roots infected by *Fusarium oxysporum* f.sp. *cubense* tropical race 4. Euphytica 205: 837-849. DOI : 10.1007/s10681-015-1418-z.

Liu S, Li J, Zhang Y, Liu N, Viljoen A, Mostert D, Zuo C, Hu C, Bi F, Gao H, Sheng O, Deng G, Yang Q, Dong T, Dou T, Yi G, Ma LJ, Li C. 2020. Fusaric acid instigates the invasion of banana by *Fusarium oxysporum* f. sp. *cubense* TR4. New Phytol 225 (2): 913-929. DOI: 10.1111/nph.16193.

Magdama F, Maggi LM, Serrano L, Sosa D, Geiser DM, Jiménez-Gasco MM. 2019. Comparative analysis uncovers the limitations of current molecular detection methods for *Fusarium oxysporum* f.sp. *cubense* race 4 strains. PLoS ONE 14 (9): 1-17. DOI:10.1371/journal.pone.0222727.

Maldonado BLD, Villarruel OJL, Calderón OMA, Sánchez EAC. 2018. Secreted in xylem (six) genes in *Fusarium oxysporum* f.sp. *cubense* and their potential acquisition by horizontal transfer. Adv Biotech Micro 10 (1): 11-18. DOI: 10.19080/AIBM.2018.09.555775.

Malik A, Preety. 2019. Purification and properties of plant chitinases: A review. J Food Biochem 43: 1-11. DOI: 10.1111/jfbc.12762.

Metsalu T, Vilo J. 2015. ClustVis: a web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap. Nucleic Acids Res 43: W566-W570. DOI: 10.1093/nar/gkv468.

Poon NK, Teo CH, Othman RY. 2019. Differential gene expression analysis of Secreted in Xylem (SIX) genes from *Fusarium oxysporum* f.sp. *cubense* tropical race 4 in *Musa acuminata* cv. Berangan and potential application for early detection of infection. J Gen Plant Pathol 86: 13-23. DOI: 10.1007/s10327-019-00882-6.

Pratama Y, Wibowo A, Widiastuti A, Subandiyah S, Widinugraheni S, Rep M. 2018. Evaluation of some specific primer sets development for detecting *Fusarium oxysporum* f.sp. *cubense* Tropic Race 4 (*Foc* TR4) originating from Indonesia. Jurnal Perlindungan Tanaman Indonesia 22 (2): 82-90. DOI: 10.22146/jpti.25037. [Indonesian]

Rep M, van der Does HC, Meijer M, van Wijk R, Houterman PM, Dekker HL, de Koster CG, Cornelissen BJ. 2004. A small, cysteine-rich protein secreted by *Fusarium oxysporum* during colonization of xylem vessels is required for I-3-mediated resistance in tomato. Mol Microbiol 53 (5): 1373-1383. DOI: 10.1111/j.1365-2958.2004.04177.x.

Rocha AJ, Soares JMDS, Nascimento FDS, Rocha ADS, Amorim VBO, Ramos APS, Ferreira CF, Haddad F, Amorim EP. 2022. Molecular, histological and histochemical responses of banana cultivars challenged with *Fusarium oxysporum* f.sp. *cubense* with different levels of virulence. Plants 11: 1-23. DOI: 10.3390/plants11182339.

Subramaniam S, Maziah M, Sariah M, Puad MP, Xavier R. 2006. Bioassay method for testing *Fusarium* wilt disease tolerance in transgenic banana. Sci Hortic 108: 378-389. DOI: 10.1016/j.scienta.2006.02.028.

Sutherland R, Viljoen A, Myburg AA, Van den Berg N. 2013. Pathogenecity associated genes in *Fusarium oxysporum* f.sp *cubense* race 4. S Afr J Sci 109: 1-9. DOI: 10.1590/sajs.2013/20120023.

Swarupa V, Ravishankar KV, Rekha A. 2014. Plant defense response against *Fusarium oxysporum* and strategies to develop tolerant genotypes in banana. Planta 239: 735-751. DOI: 10.1007/s00425-013-2024-8.

Vaghela B, Vashi R, Rajput K, Joshi R. 2022. Plant chitinases and their role in plant defense: A comprehensive review. Enzyme Microb Technol 159: 110055. DOI: 10.1016/j.enzmictec.2022.110055.

van der Does HC, Duyvesteijn RG, Goltstein PM, van Schie CC, Manders EM, Cornelissen BJ, Rep M.. 2008. Expression of effector gene SIX1 of *Fusarium oxysporum* requires living plant cells. Fungal Genet Biol 45 (9): 1257-1264. DOI: 10.1016/j.fgb.2008.06.002.

Wang D, Peng C, Zheng X, Chang L, Xu B, Tong Z. 2020. Secretome analysis of the banana *Fusarium* wilt fungi *Foc R1* and *Foc TR4* reveals a new effector OASTL required for full pathogenicity of *Foc TR4* in banana. Biomolecules 10: 1-17. DOI: 10.3390/biom10101430.

Wibowo A, Subandiyah S, Sumardiyono C, Sulistyowati L, Taylor P, Fegan M. 2011. Occurence of Tropical Race 4 *Fusarium oxysporum* f.sp. *cubense* in Indonesia. Plant Pathol J 27: 280-284. DOI: 10.5423/PPJ.2011.27.3.280.

Widinugraheni S, Niño-Sánchez J, van der Does HC, van Dam P, García-Bastidas FA, Subandiyah S, Meijer HJG, Kistler HC, Kema GHJ, Rep M. 2018. A *SIX1* homolog in *Fusarium oxysporum* f.sp. *cubense* tropical race 4 contributes to virulence towards Cavendish banana. PLoS ONE 13 (10): e0205896. DOI: 10.1371/journal.pone.0205896.

Yanti Y, Warnita, Reflin, Busniah M. 2018. Indigenous endophyte bacteria ability to control *Ralstonia* and *Fusarium* wilt disease on chili pepper. Biodiversitas 19: 1532-1538. DOI: 10.13057/biodiv/d190446.

Zhang L. Cenci A. Rouard M, Zhang D, Wang Y, Tang W, Zheng S.J. 2019. Transcriptomic analysis of resistant and susceptible banana corms in response to infection by *Fusarium oxysporum* f.sp. *cubense* tropical race 4. Sci Rep 9: 1-14. DOI: 10.1038/s41598-019-44637-x.