



Original Article

# *In vitro* characterization of banana cultivar rejang responses to *Fusarium oxysporum* f. sp. *ubense* tropical race 4 for agricultural biosecurity risk assessment

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**Abstract**—*Fusarium oxysporum* is a highly destructive soilborne pathogen comprising more than 100 host-specific strains (*formae speciales*), making it a significant biosecurity threat to many economically important crops, including tomato, melon, cotton, and banana. Its detrimental impact on agricultural productivity poses serious challenges to food security and the sustainability of major agricultural commodities. Understanding the infection mechanism of *Fusarium oxysporum* f.sp. *ubense* Tropical Race 4 (*Foc* TR4) and the defense responses of banana plants against this pathogen is essential for developing effective disease management strategies and enhancing resistance to Fusarium wilt. This study aimed to analyze the expression of the fungal virulence genes *SIX6* and *SIX9*, as well as pathogenesis related genes *PR-1* and *PR-3*, in the roots, corms, and leaves of banana plants infected with *Foc* TR4. The results showed that tissue-cultured banana cv. Rejang was highly susceptible to *Foc* TR4 infection. In this susceptible cultivar, the expression of pathogenesis-related genes (*PR-1* and *PR-1*) was markedly elevated in roots in response to the expression of virulence expressing genes (*SIX6* and *SIX9*), whereas lower expression levels were observed in corms and leaves. These findings provide valuable insights into the interaction between *Foc* TR4 and banana defense mechanisms and may serve as a foundation for the development of Fusarium wilt-resistant banana cultivars, thereby contributing to agricultural biosecurity and food security.

**Keywords**—*Foc* TR4; Fusarium wilt; In vitro culture; *PR* genes expression; Rejang; *SIX* genes expression;

## 1. INTRODUCTION

After rice, wheat, and corn, banana plants become the fourth important export commodity worldwide. As one of the most sought-after agricultural commodities, Indonesian bananas have established a significant role in supporting international market activities [1], with production showing an increasing trend from 7.26 million tons in 2018 to 9.81 million tons in 2025 [2]. The continuity of banana production is threatened by pests and diseases. One of the devastating factors is the soilborne pathogen *Fusarium oxysporum* f.sp. *ubense* (*Foc*), the causal agent of Fusarium wilt.

*Fusarium oxysporum* is a ubiquitous and highly destructive soilborne pathogen, predominantly represented by host-specific strains referred to as *formae speciales*. Although at least 130 *formae speciales* of *F. oxysporum* have been documented in the Index Fungorum and MycoBank databases as of January 2025. A recent investigation identified 46 *Fusarium* species associated with wilt diseases, including seven newly described species, with several species exhibiting the ability to infect multiple hosts, including tomato, cucumber, strawberry, banana, pepper, and eggplant [3]. Recent evidence indicates that Fusarium wilt disease has

spread across several key banana-producing areas in Indonesia, such as Bengkulu [4], Banten [5], and Central Java [6]. Fusarium wilt disease has become widely distributed throughout Indonesia, with pathogen populations consisting of *Foc* Race 1 (*FocR1*), Race 4 (*FocR4*), and Tropical Race 4 (TR4) over the past two decades [7].

One of the important effectors found in *Foc* TR4 is the secreted in xylem (*SIX*) proteins [3]. Initially, these effectors were found in *F. oxysporum* f.sp. *lycopersici* (*Fol*) that infects tomato [8, 9, 10]. *SIX3* (also known as *Avr2*) contributes to *Fol* virulence in susceptible hosts and triggers resistance in tomato plants carrying the I-2 resistance gene [10]. Furthermore, the interaction between *SIX5* and *SIX3* (*Avr2*) is required for the activation of I-2 resistance, while mutations in *SIX5* enable the pathogen to evade host recognition and simultaneously reduce its virulence [9]. Up to now, 14 effectors have been identified in banana and *SIX* gene homologs have been found in *F. oxysporum* infecting other plants [10]. In Cavendish, *SIX6*, *SIX8*, *SIX9* was reported to be involved in virulence of *Foc* TR4 in numerous studies [11, 12, 13, 14]. However, their role in

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pathogenicity in banana plants has not been determined. Understanding the mechanism of *Foc* infection and how banana counteracts the pathogen can be useful in the development of resistance towards Fusarium wilt.

Banana cv. Rejang (*Musa acuminata*, AA genome) is well known for its resistance to *Foc* TR4 infection [15]. However, under tissue culture conditions, this cultivar was found to be highly susceptible to *Foc* TR4. Furthermore, in this susceptible cultivar, the expression of pathogenesis-related (*PR*) genes was considerably elevated in roots in response to the expression of virulence-expressing genes *SIX* genes, whereas lower expression levels were observed in corms and leaves. These findings provide valuable insights into the interaction between banana and *Foc* TR4 and may support the development of resistant cultivars and early disease detection strategies, thereby strengthening agricultural biosecurity and mitigating biological threats within the CBRNE framework.

## 2. MATERIALS AND METHODS

### 2.1. Plant Materials and Pathogen Inoculation

*Foc* TR4 was isolated from infected banana cv. Bading kayu susu, also known as biu kayu which is unique to Bali. The molecular identity of *Foc* isolate was confirmed by PCR using the primer pairs ITS5/ITS4 [16], EF1/EF2 specific for the genus *Fusarium* [17], and *Foc*TR4-specific primers [18]. All sequences were compared to the GenBank database using BLAST ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)).

*Musa acuminata* cv. Rejang (AA group) plantlets were propagated in Murashige and Skoog (MS) media containing 2.5 ppm of benzyl amino purine (BAP). Plantlets with 3–5 leaves were selected for inoculation with *Foc* TR4. Banana cultivars were grown in a controlled room with temperature and light intensity. *Foc* isolate was grown in Potato Dextrose Agar (PDA) media for 7 days at room temperature and prepared as a suspension of  $10^6$  spore/ml in 0.85 % NaCl. Plantlets were acclimatized 2 days prior infection in MS ½ and inoculated with 1 ml of *Foc* suspension and incubated on a 85 rpm shaker at room temperature with 16:8 photoperiod for 14 days.

### 2.2. Disease Severity Index

Fourteen days post infection, banana plantlets were observed for disease severity using leaf symptom index (LSI) and rhizome discoloration index (RDI) [19]. LSI was determined as follows: 1) no yellow and wilting on the leaf; 2) small bit of yellowing observed on the outer leaf; 3) yellow and wilting observed on the outer leaf, young leaf appeared to yellow; 4) most leaf are yellow and wilting; 5) dead plants. RDI was determined as follows: 1) no discoloration observed on the xylem and its surrounding; 2) no discoloration on xylem, but discoloration observed at the junction of root and pseudostem; 3) up to 5 % discoloration on xylem and its surrounding; 4) 6–20 % of discoloration on xylem and its surrounding; 5) 21–50 % of discoloration on xylem and its surrounding; 6) more

than 50 % discoloration on xylem and its surrounding; 7) discoloration on the entire xylem and its surrounding; 8) dead plant. The overall disease severity index (DSI) for LSI and RDI was calculated by Eq. (1).

$$DSI = \frac{\sum (\text{number on scale} \times \text{number of seedlings in that scale})}{\sum \text{number of treated seedlings}} \quad (1)$$

Its interpretation was determined based on the following criteria: Resistance if DSI scale for LSI and RDI is 1 for each; Tolerant if DSI scale for LSI is between 1.1 and 2 and RDI is between 1.1 and 3; Susceptible if LSI is between 2.1 and 3 and RDI is between 3.1 and 5; Highly susceptible if LSI is between 3.1 and 4 and RDI is between 5.1 and 8. If the cultivar is resistance in LSI and tolerant in RDI, the cultivar is considered as tolerant. If LSI is susceptible and RDI is tolerant, the cultivar is susceptible. When both LSI and RDI are resistance, the cultivar is resistance. When one of them is tolerant, the cultivar is considered as tolerant.

### 2.3. RNA Extraction and Quantitative Real-time PCR

Total RNAs were isolated from the roots, corms, and leaves of the *Foc* TR4 infected banana cv. Rejang 3, 6, 9 and 14 dpi. First-strand cDNA synthesis was performed with 1 gram of total RNA employing the iScript cDNA synthesis kit according to the manufacturer's instruction (Biorad, California, USA). The expression levels of the *SIX6* and *SIX9* genes, as well as the *PR-1* and *PR-3* genes, were quantified using GoTaq® qPCR Master Mix (Promega, Wisconsin, USA) on a QuantStudio 1 Real-Time PCR System (Applied Biosystems, California, USA). Gene expression data were normalized against the reference gene *GAPDH* [20] and calculated using the  $2^{-\Delta\Delta Ct}$  method [21]. Three replicates of each sample were analyzed to ensure reproducibility and reliability.

## 3. RESULT AND DISCUSSION

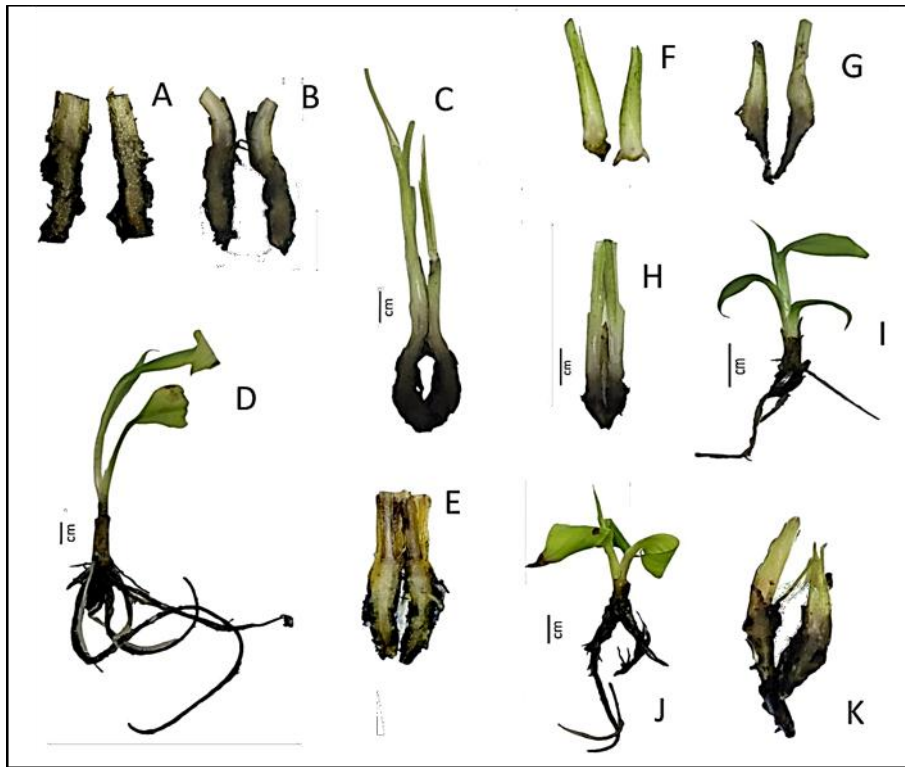
### 3.1. Banana Plantlets cv. Rejang is Highly Susceptible to *Foc* TR4

Banana plantlets cv. Rejang were infected with *Foc* TR4 and observed 3, 6, 9 and 14 days post infection (dpi) (**Figure 1**). Cavendish is well known being susceptible to *Foc* TR4, hence become an experimental control. In cv. Rejang, browning of the corm can be observed from 3 dpi (**Figure 1A**) whereas in Cavendish, the browning can be observed from 6 dpi (**Figure 1G**). After 14 dpi, leaf symptom index (LSI) and rhizome discoloration index (RDI) were calculated in both cultivars. As shown in **Table 1**, Cavendish was categorized as highly susceptible with LSI 3.7 and RDI 5.9. Similar to Cavendish, Rejang also categorized as highly susceptible with LSI 3.8 and RDI 7.

**Table 1.** Disease index of cv. Rejang

Cultivar	LSI	RDI	Category
Cavendish	3.7	5.9	Highly susceptible
Rejang	3.8	7	Highly susceptible

LSI: leaf symptom index; RDI: Rhizome Discoloration Index. The experiments were conducted in two independent tests with similar results.



**Figure 1.** Pathogenicity assay on banana cv. Rejang (A to E) and Cavendish (F to K) infected with *Foc* TR4. A) Infected corm 3 dpi; B) Infected corm 6 dpi; C) Infected corm and pseudostem 9 dpi; D) Infected plantlet 14 dpi; E) Corm and lower pseudostem of infected plantlet 14 dpi; F) Infected corm 3 dpi; G) Infected corm 6 dpi; H) Infected corm and pseudostem 9 dpi; I) Infected plantlet 9 dpi; J) Infected plantlet 14 dpi; K) Infected corm and pseudostem 14 dpi.

### 3.2. *SIX* and *PR* Genes Expression in Bananas during Infection with *Foc* TR4

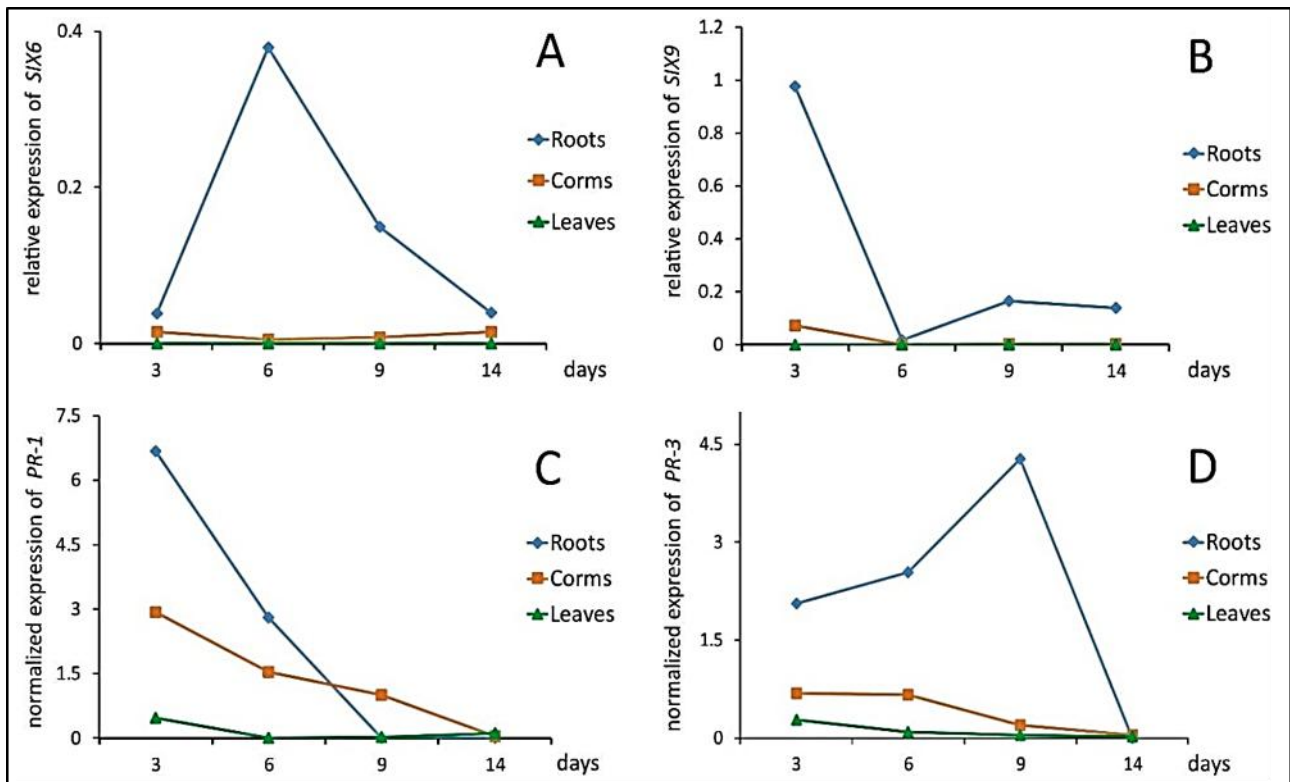
*SIX6* and *SIX9* genes are highly expressed in roots of infected bananas, but not in corms nor the leaves (**Figure 2A** and **2B**). In Rejang roots, the expression of *SIX6* gene was elevated as high as 0.38-fold at 6 days post infection (dpi), whereas *SIX9* was 0.98 at 3 dpi. During infection, the expression of pathogenesis-related (*PR*) genes *PR-1* and *PR-3* in susceptible cultivar Rejang was examined. *PR-1* was expressed early in the roots 3 dpi with 6.67-fold expression (**Figure 2C**) whereas in *PR-3*, the highest expression was reached in roots 9 dpi with 4.27-fold expression. In corms, the highest expression of *PR-3* was reached at 3 dpi with 0.68-fold expression (**Figure 2D**). The expression of both genes was considerably low in the leaves.

The never-ending battle between pathogens and hosts leads to co-evolutionary arms race where both evolve to counteract each other [22]. A successful pathogen must be able to maintain the ability to avoid host recognition but still virulent in the process. This will determine infectivity and host specialization. In order to do this, pathogens will have to pass a series of gene modifications, changes in the expression of existing effector genes or even generate new effectors [23]. Generally, effectors are modular proteins. They contain signal peptides, relatively small in size, rich in cysteine residues and do not have similarity with known proteins [8]. In host cells, effectors may suppress host defense systems or deceive host cells to accommodate further infection and colonization [24]. Fungal pathogens have

developed the ability to deliver effectors inside the host cytoplasm as well as in the extracellular space, thus classified as cytoplasmic and apoplastic effectors, respectively [25].

*Fusarium oxysporum* has attracted plant pathologists across the globe due to its devastating impact on economy of many countries and also because of its evolutionary quests affecting different hosts, hence the name *Fusarium oxysporum* species complex (FOSC) [26]. The soil-borne fungus in FOSC includes both nonpathogenic and pathogenic strains [26]. In banana and many other important crops, the pathogenic strains invade roots and causes wilting via colonization of xylem tissue [24]. More than 120 *formae speciales* (ff.spp.) have been identified in pathogenic *Fo* strains [3]. The *formae speciales* refers to narrow host specificity, where each *forma specialis* infects specific plant species [3]. However, this host range was subsequently found to be wider in many *formae speciales* not only in plants but also in humans [3,27]. The *Fo* pathogenic strains usually are hemi-biotrophs, performing a biotrophic lifestyle at early stages of infection and at later stages release toxins in order to kill the host cells and obtain nutrients on the dead tissue [28].

The co-evolutionary arm race between pathogens and hosts can be observed in an interplay between genes involved in the interaction, namely resistance (*R*) genes in host plants and avirulence (*Avr*) genes in pathogens [25, 29]. *Avr* genes are known as effectors that have the ability to manipulate the host immune system to avoid detection and optimizing the virulence function [28]. Host



**Figure 2.** Gene expression of *SIX6*, *SIX9*, *PR-1* and *PR-3* in cv. Rejang after infection with *Foc TR4*. (A) Relative expression of *SIX6* and (B) *SIX9* gene in roots, corms and leaves of Rejang 3, 6, 9, and 14 days post infection (dpi) with *Foc TR4*. (C) Normalized expression of *PR-1* and (D) *PR-3* gene in roots, corms and leaves of Rejang 3, 6, 9, and 14 dpi with *Foc TR4*. The *GAPDH* was used as a reference gene.

plants evolved by recognizing these specific proteins via R genes [22]. *SIX* genes have been reported involved in virulence and host manipulations in susceptible cultivar [25]. However, in resistant cultivars, these genes mediated and triggered resistance. *SIX6* was reported to contribute in virulence of *Fol* and suppress I-2-mediated cell death [30]. However, its role in *Foc*-banana pathosystem has not been determined yet. To overcome the fungal attack, the host cells were expressing the pathogenesis-related (*PR*) genes which are crucial components of plant innate immune system especially systemic acquired resistance, thus extensively utilized as markers for defense signaling pathways [31]. The overexpression of *PR-1* gene was reported enhance resistance in plants during bacterial and fungal attacks [13]. *PR-3* gene encodes a chitinase that disintegrate chitin in fungal cell walls and inhibit the fungal growth [13, 30] In susceptible banana cultivar, these *PR* genes were highly expressed in the roots and subsequently in the corms (Figure 2C and D).

#### 4. CONCLUSION

Tissue-cultured banana cv. Rejang exhibited a high susceptibility to *Foc TR4* infection. In this susceptible cultivar, the expression of pathogenesis-related (*PR*) genes was significantly elevated in roots in response to virulence-expressing genes *SIX* genes, whereas lower expression levels were observed in corms and leaves. These findings provide valuable insights into the molecular interactions between banana and *Foc TR4*,

supporting the development of resistant banana cultivars and serving as a basis for early disease surveillance strategies to anticipate and mitigate biological threats that may affect the stability and sustainability of food production and strategic agricultural commodities within the CBRNE framework.

#### 5. AUTHOR'S DECLARATION

##### 5.1. Supporting Information

No additional supporting information accompanies this manuscript. All experimental procedures, data analyses, and research findings relevant to the objectives of this study have been adequately described and presented in the main text. The qPCR datasets generated and analyzed during this study can be made available upon request.

##### 5.2. Acknowledgements

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### 5.3. Conflict of Interest

The authors confirm that no conflicts of interest exist regarding the preparation, submission, and publication of this manuscript.

### 5.4. Author Contributions

NF, RRE, and FMD contributed to the conception and design of the study. PB performed the experiments, collected and analyzed the data, and prepared the initial draft of the manuscript. RH collected and analyzed the data. NF supervised the research, contributed to data interpretation, and provided critical revisions to the manuscript. All authors reviewed, approved, and agreed to the published version of the manuscript.

### 5.5. AI Statement

Artificial intelligence (AI) tools were used solely to assist with language editing, grammar improvement and graphical abstract design. All scientific content, analyses, interpretations, and conclusions were developed and verified by the authors, who assume full responsibility for the content of this manuscript.

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