Journal of Plant Protection Research eISSN 1899-007X

ORIGINAL ARTICLE

Evaluation of banana cultivars and the pathogenesis-related class 3 and 10 proteins in defense against *Ralstonia syzygii* subsp. *celebesensis*, the causal agent of banana blood disease

Jariya Nitayaros[1], Thanwanit Thanyasiriwat[1], Aphidech Sangdee[2], Ladawan Rattanapolsan[1], Ratri Boonruangrod[3], Praphat Kawicha[1]*

[1] Plant Genome and Disease Research Unit, Department of Agriculture and Resources, Faculty of Natural Resources and Agro-Industry, Kasetsart University, Chalermphrakiat Sakon Nakhon Province Campus, Sakon Nakhon, Thailand

[2] Department of Biology, Mahasarakham University, Faculty of Science, Maha Sarakham, Thailand

[3] Department of Horticulture, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University Kamphaeng Saen Campus, Nakhon Pathom, Thailand

Vol. 63, No. 3: 375–386, 2023

DOI: 10.24425/jppr.2023.146873

Received: April 27, 2023
Accepted: July 25, 2023
Online publication: September 04, 2023

*Corresponding address:
csnppkc@ku.ac.th

Responsible Editor:
Joanna Puławska

Abstract

Banana blood disease (BBD), caused by *Ralstonia syzygii* subsp. *celebesensis* (*Rsc*), is a major threat to banana production in Southeast Asia. This study aimed to assess the resistance of cultivated and wild banana accessions to *Rsc* and investigate the expression of pathogenesis-related (PR) protein genes, namely *PR3* and *PR10*, in disease-resistant bananas. Bacterial isolates were isolated from infected bananas in Yala Province, Thailand, and their pathogenicity and phylotype were confirmed, along with *Rsc*-specific PCR. *Rsc*-resistance banana screening was conducted on 16 banana accessions, including cultivated and wild types, using representative *Rsc* isolates. 'Khai Kasetsart 2' exhibited resistance (R), followed by 'Raksa' with moderate resistance (MR). The expression of *PR3* and *PR10* genes was analyzed in the resistant 'Khai Kasetsart 2' and susceptible 'Hin' bananas, revealing distinct expression patterns. *PR3* showed rapid upregulation on day 1 after inoculation (DAI), while *PR10* exhibited sustained upregulation from 1 to 7 DAI in the resistant cultivar. These findings indicate the involvement of PR proteins in the defense response against *Rsc* and hold promise for future breeding and disease management strategies in bananas.

Keywords: banana, blood disease, gene expression, *PR* gene, *Ralstonia*, resistance

Introduction

Banana blood disease (BBD) is caused by *Ralstonia syzygii* subsp. *celebesensis* (*Rsc*) (Safni *et al*. 2014). It was first reported in southern Sulawesi, Indonesia, in the early 1900s and has since spread to Indonesia, Papua New Guinea, and Malaysia (Davis *et al*. 2001; Safni *et al*. 2014; Teng *et al*. 2016). BBD may be a severe threat to banana production because of its increasing frequency in different regions of the world and its effect in reducing crop yield in addition to increasing the cost of disease management programs (Blomme *et al*. 2017). Infected banana plants are unable to produce edible fruits, resulting in substantial yield losses (Supriadi 2005).

The transmission of BBD occurs through infected banana propagative materials, insect mechanical transmission, and contaminated agricultural tools, soil, and water (Buddenhagen 2009). To control the spread of BBD, current measures involve primary quarantine and sanitation practices (Davis *et al*. 2001). However, certain banana cultivars have shown

tolerance to BBD, making them potential genetic resistance sources for breeding programs (Supriadi 2005).

Understanding the plant immune response to disease-causing agents is crucial for developing effective disease control strategies. When plants are infected by pathogens, they activate their immune system through signaling pathways that lead to the expression of defense response genes (Andersen *et al.* 2018). This activation involves the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) on the cell membrane and the detection of cellular damage-associated molecular patterns (DAMPs) by wall-associated kinases (WAKs) (Zipfel 2014; Decreux and Messiaen 2005). Pathogen effectors are recognized by receptors with nucleotide-binding domains and leucine-rich repeats (NLRs) (Dangl *et al.* 2013). These processes trigger various response mechanisms, including the hypersensitive response (HR), production of reactive oxygen species (ROS), cell wall modification, and synthesis of pathogenesis-related (PR) proteins.

PR proteins, such as oxidases and oxidase-like proteins (PR-9, 15, and 16), chitinases (PR-3, 4, 8, and 11), β-1,3-glucanases (PR-2), endoproteinases (PR-7), proteinase inhibitors (PR-6), lipid-transfer proteins (PR-14), ribonuclease-like proteins (PR-10), defensins (PR-12), thionins (PR-13), and thaumatin-like proteins (PR-5), are defense-related proteins that play a role in limiting the invasion of plant pathogenic fungi (van Loon 1999; Osmond *et al.* 2001; Ebrahim *et al.* 2011; Sinha *et al.* 2014) and bacteria (Tang *et al.* 2017). However, our knowledge of PR proteins against plant pathogenic bacteria remains limited.

In this study, we aimed to screen BBD-resistant cultivars through pot experiments to identify potential genetic sources of resistance. We analyzed and compared the expressions of *PR3* and *PR10* protein genes using real-time quantitative PCR between resistant and susceptible banana cultivars. By studying the interactions between the BBD pathogen and *Rsc*-resistant cultivars, the findings can provide valuable insights into the development of effective strategies for managing BBD in bananas.

Materials and Methods

Preparation of banana cultivar samples

The banana accessions used in this study were obtained from the banana germplasm collections of Kasetsart University, Thailand (Table 1). Sixteen banana accessions were propagated through micropropagation. Banana shoot explants were subcultured in Murashige and Skoog (MS) medium (Murashige and Skoog 1962) amended with 5 mg · l^{-1} BAP (6-benzylaminopurine) every month for 4 months. The multiplied shoots were then divided and transferred to MS medium to facilitate root development for 1 month. The micropropagated plants were subsequently transferred to peat moss-filled seedling trays and acclimatized in a controlled greenhouse at a temperature of 30°C and humidity of 60% for 1 month. Following acclimatization, the plants were transferred to potting soil in 3 × 6 inch grow bags and placed in a greenhouse for 2 months. The plants were watered once a day with 50 ml of tap water per plant and received 1 g of 15-15-15 fertilizer every 10 days until the end of the experiment.

Isolation and pathogenicity of the pathogen

Diseased plants showing typical symptoms of banana blood disease were collected from Yala Province, Thailand. The BBD pathogen was isolated from banana plants exhibiting characteristic symptoms such as leaf yellowing, petiole collapsing, leaf wilting, reddish-brown discoloration in vascular bundles, and discolored reddish-brown and rotten fruit pulp. To ensure surface disinfection, banana fruits were dipped in a 3% sodium hypochlorite solution for 5 minutes. The fruits were then crosscut, and a 0.5 × 0.5 cm explant from the epicarp was taken. The explant was aseptically cut into small pieces and placed in a microcentrifuge tube containing 100 µl of 1X phosphate-buffered saline (PBS) for 30 minutes. The suspension was streak-plated on triphenyl tetrazolium chloride (TZC) media (Kelman 1954) plates and incubated at 28°C for 4 days.

A single colony was selected, and its pathogenicity was confirmed using Koch's postulates. The selected colony was cultured on casamino acid-peptone-glucose (CPG) media (Kelman 1954) at 28°C for 3 days. The bacterial cells were collected by scraping and suspended in phosphate-buffered saline (PBS) solution. The concentration of bacterial cells was adjusted to 10^8 CFU · ml^{-1}. Ten milliliters of the bacterial suspension were inoculated into 3-month-old 'Hin' bananas, prepared as described earlier, through two sites of wounded roots. The wounded roots were created by stabbing a cutting blade (18 mm wide) 5 cm below the soil surface and 2 cm from the banana stem. One month after inoculation, disease symptoms were observed, and the bacterial pathogen was reisolated. The reisolated bacteria were confirmed for their pathogenicity again using the same inoculation procedure. The pathogenicity assessment was performed with 10 replications. *Ralstonia solanacearum* isolates 832, 1350, and 1481, derived from the Department of Agriculture, Thailand, were compared. The disease severity score (DSS) was rated on a 0–5 scale, according to Bakar *et al.* (2018). The DSS was scored using a five-grade severity scale: 0 – symptomless, 1 – wilted leaves,

Table 1. List of banana cultivars used for evaluating *Rsc*-resistance bananas

Type	Species/Group*	Cultivar name	Accession no.	Source
Wild type	*Musa acuminata*	–	HB203	KU KPS
			HB210	KU KPS
			HB220	KU KPS
			HB247	KU KPS
Cultivar	AA	'Khai Kasetsart 2'	BK002	KU
		'Raksa'	HB024	KU KPS
	AAA	'Nio Jorakhe'	BK003	KU
		'Hom Khiew Khom'	HB132	KU KPS
			HB237	KU KPS
		'Krang'	HB227	KU KPS
		'Hom Thong Pa'	HB239	KU KPS
		'Hom Africa'	HB240	KU KPS
		'Hom Thong'	HB243	KU KPS
		'Hom Taiwan'	BK004	KU
	ABB	'Hin'	Hin-CI	KU CL
		'Namwa Mali-Ong'	BK005	KU

*the informal nomenclature system used to classify banana cultivars was developed by Simmonds and Shepherd (1955); AA – the AA genome group comprises cultivars with two sets of chromosomes inherited from *Musa acuminata*; AAA – the AAA genome group includes cultivars with three sets of chromosomes inherited from *M. acuminata*; ABB – the ABB genome group comprises cultivars with one set of chromosomes donated by *M. acuminata* and two by *M. balbisiana*. KU CL – Central Laboratory and Greenhouse Complex, Research and Service Center, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Kamphaeng Saen Campus, Thailand; KU – Department of Horticulture, Faculty of Agriculture, Kasetsart University, Thailand; KU KPS – Department of Horticulture, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Kamphaeng Saen Campus, Thailand

2 – initial yellowing, 3 – 2 to 3 chlorotic leaves, 4 – 4 or more chlorotic leaves, and 5 – plant death. Disease incidence percentage (DI) was calculated by dividing the total number of diseased plants by the total number of inoculated plants.

Identification of the pathogen

The pathogenic bacterial isolates were identified for their phylotype using a multiplex PCR method described by Fegan and Prior (2005), employing the primers listed in Table 2. To confirm that the isolated bacterium was *Rsc*, the BDB-specific PCR developed by Das (2004) was conducted using the 121F and 121R primers listed in Table 2. *Ralstonia solanacearum* isolates 832, 1350, and 1481 were included in the comparison. The bacteria were cultured on CPG media at 28°C for 2 days. Subsequently, bacterial cells were collected and suspended in PBS buffer. Genomic DNA extraction was performed using the GF-1 Bacterial DNA Extraction Kit (Vivantis, Malaysia). The extracted genomic DNA served as a template for the PCR reaction, where a 25 µl reaction mixture contained 50 ng of bacterial genomic DNA, 1X OnePCR™ Ultra (GeneDirex, Taiwan), 0.4 µM of the forward primer, and 0.4 µM of the reverse primer (primer sequences provided in Table 2). The PCR was conducted using a thermocycler (PCRmax Alpha cyclers, AC-1, UK) with the following thermal cycling profile: initial denaturation at 96°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 45 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 45 seconds, and a final extension step at 72°C for 5 minutes. The PCR products were separated on a 1.5% agarose gel through electrophoresis at 100 volts for 45 minutes.

To identify the representative bacterial isolates, BT4101 and MY4101, a partial sequence of the 16S ribosomal RNA (16S rRNA) gene was analyzed. The PCR reactions followed a protocol similar to the previous amplification, with modifications in using primers specific to the 16S rRNA gene (Lane 1991) (details provided in Table 2). The thermal cycling profile was adjusted accordingly, and the annealing temperature is specified in Table 2. Following amplification, the PCR products were purified using the BioFact™ PCR Purification Kit (Biofact, South Korea). Subsequently, the resulting sequences were sent to Macrogen (Korea) for analysis. Low-quality sequences were trimmed using Bioedit version 7.2.5, and the approved sequences were compared with reference sequences available in the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST). A phylogenetic tree was constructed to visualize the relationship between the selected bacteria and the reference sequences using MEGA X version 10.0.5.

Table 2. Primer sequences for the identification of isolated bacteria

Gene/Target	Primer name	Sequence (5′→ 3′)	Product size [bp]	Annealing [°C]	Reference
Phylotype--specific multiplex PCR	Nmult21:2F (Phylotype II)	CGTTGATGAGGCGCGCAATTT	144*	60	Fegan and Prior (2005)
	Nmult21:1F (Phylotype I)	AAGTTATGGACGGTGGAAGTC	372*		
	Nmult23:AF (Phylotype III)	ATTACSAGAGCAATCGAAAGATT	91*		
	Nmult22 : InF (Phylotype IV)	ATTGCCAAGACGAGAGAAGTA	213*		
	Nmult22:RR (All Phylotype)	TCGCTTGACCCTATAACGAGTA			
	759F 760R	AGAGTTTGATCMTGGCTCAG TACGGYTACCTTGTTACGACTT	280**		Opina *et al.* (1997)
BDB-specific PCR	121F	CGTATTGGATGCCGTAATGGA	317	60	Das (2004)
	121R	AAGTTCATTGGTGCCGAATCA			
16S rRNA	27F	AGAGTTTGATCMTGGCTCAG	1,450	58	Lane (1991)
	1492R	TACGGYTACCTTGTTACGACTT			

*amplicon size when paired with the reverse primer Nmult:22:RR., **amplicon size of the target sequences amplified from *Ralstonia* spp. using primers 759F and 760R. The amplicon sizes for *Ralstonia* spp. Phylotype I are 144 bp and 280 bp. For *Ralstonia* spp. Phylotype II, the amplicon sizes are 372 bp and 280 bp. *Ralstonia* spp. Phylotype III has amplicon sizes of 91 bp and 280 bp, while *Ralstonia* spp. Phylotype IV has amplicon sizes of 213 bp and 280 bp

Evaluation of *Rsc*-resistant banana cultivars

Sixteen banana accessions, consisting of four wild-type accessions and 12 banana cultivars (as shown in Table 1), were subjected to inoculation with bacterial suspensions of *Rsc* isolates BT4101 and MY410. The preparation of test plants and inoculums was described earlier. Four weeks post-inoculation, the disease severity score (DSS) and disease severity index (DSI) were assessed and compared to the susceptible 'Hin' banana. The experiments were conducted with 10 replicates. The DSS was evaluated on a 0–5 scale, following the rating system of Bakar *et al.* (2018), where: 0 – indicated no symptoms, 1 – represented wilting of leaves, 2 – denoted initial yellowing, 3 – indicated 2 to 3 chlorotic leaves, 4 – represented 4 or more chlorotic leaves, and 5 – signified plant death. The DSI was calculated using the following formula:

$$\mathrm{DSI} = \frac{\sum S_i \times N_j}{S \times N_t} \times 100,$$

where:
S_i – the disease severity score,
N_j – the number of tested plants with severity score,
S – the highest disease severity score,
N_t – the total number of tested plants.

The DSI score was used to determine the disease resistance type of banana cultivars (DSI ≤ 5% = R, resistance; DSI < 50% = MR, moderate resistance; DSI > 50% = S, susceptible; 100% DSI = HS, highly susceptible).

Measurement of pathogenesis-related protein gene expression

The resistant banana cultivar 'Khai Kasetsart 2' and susceptible banana cultivar 'Hin' were inoculated with *Rsc* isolate MY4101. Plant samples, consisting of 5 cm of pseudostem above the ground, were collected 1, 3, 7, and 14 days after inoculation (DAI). Total RNA was extracted using the RNeasy Kit (QIAGEN, Germany), and the RNA was then used to synthesize complementary DNA (cDNA) using the Viva cDNA Synthesis Kit (Vivantis, Malaysia). The expression levels of pathogenesis-related protein genes, including *PR3* and *PR10* genes, were analyzed. The housekeeping gene *β-Actin* was used as an internal reference. The primer

Table 3. Primer sequences for Pathogenesis-related protein (*PR*) genes expression analysis

Gene/Putative function		Sequence (5′→ 3′)	Reference
PR3	forward primer	GAGGATGTGTGCTGAAGGTGGTG	Li *et al.* (2015)
	reverse primer	CTTGATGATTCCCGTCACAGTCTC	
PR10	forward primer	GTCACCACCAACATCATCAA	
	reverse primer	CCAGCAAGTCGCAGTACCTC	
β-*Actin*	forward primer	GCCATACAGTGCCAATCTACGAGG	
	reverse primer	ATGTCACGAACAATTTCCCGCTCA	

sequences used in this study were reported by Li *et al.* (2015) and are listed in Table 3. Real-time PCR reactions were performed in three technical replicates using the SensiFAST™ SYBR® No-ROX Kit (Meridian Bioscience®) and processed in the PCRmax ECO 48 REAL TIME PCR system (PCRmax Limited). The efficiency of each primer set was assessed based on its standard curve derived from a series of 2-fold diluted template cDNAs. The difference between the cycle threshold (C_t) values of the *PR* genes and the C_t value of *β-Actin* ($\Delta C_t = C_t$ target gene $- C_t$ *β-Actin*) was calculated to obtain the normalized expression of the *PR3* and *PR10* genes. The expression level was determined using the $2^{-\Delta Ct}$ method and expressed as a fold change using the $2^{-\Delta\Delta Ct}$ formula relative to a calibrator representing the expression level in mock-inoculated plants.

Results

Isolation and pathogenicity of the pathogen

Banana fruits exhibiting typical symptoms of banana blood disease (BBD) were collected from various banana plantations in Yala Province, Thailand. A total of 10 bacterial isolates were obtained, with four isolates originating from Betong district, four isolates from Mueang district, and two isolates from Raman district as indicated in Table 4. The isolated bacteria exhibited slow-growing colonies with smooth margins and dark red centers on TZC medium. When these isolated bacteria were inoculated onto 'Hin' bananas, they all caused wilt disease symptoms. The average disease severity score (DSS) measured 28 days post-inoculation ranged from 4.8 ± 0.4 to 5.0 ± 0.0. Notably, no significant variations in DSS were observed among the isolated bacteria. Furthermore, all the isolated bacteria caused disease symptoms in all tested banana replicates. In contrast, the reference isolates of *R. solanacearum* did not elicit disease symptoms in 'Hin' bananas (Table 4).

Identification of the pathogen

Based on the phylotype-specific multiplex PCR classification, all 10 pathogenic isolates were classified as phylotype IV. The PCR analysis revealed amplicon sizes of 280 bp, derived from primer 759F and 760R, and 213 bp, derived from primer Nmult22 : InF and Nmult22 : RR. The amplification product from the first primer set indicates that the pathogenic isolates belong to the *Ralstonia* species, while the product from the second primer set confirms their classification in phylotype IV. Subsequently, BDB-specific PCR was performed to amplify target sequences from the pathogenic isolates. The BDB-specific PCR successfully amplified a target size of 317 bp from all the pathogenic isolates. In contrast, all isolates of *R. solanacearum* were classified as phylotype I and tested negative in the BDB-specific PCR assay (Table 4).

Table 4. Characterization of isolated bacteria from infected bananas: pathogenicity, disease severity, phylotype, and BDB-specific PCR results

Source	Isolate	Original host	DSS	DI%	Phylotype	BDB-specific PCR
Betong district, Yala province, Thailand	BT1401	banana	4.8 ± 0.4	100	IV	+
	BT2401	banana	4.8 ± 0.4	100	IV	+
	BT3301	banana	4.9 ± 0.3	100	IV	+
	BT4101	banana	5.0 ± 0.0	100	IV	+
Mueang district, Yala province, Thailand	MY1302	banana	5.0 ± 0.0	100	IV	+
	MY2101	banana	5.0 ± 0.0	100	IV	+
	MY3501	banana	4.9 ± 0.3	100	IV	+
	MY4101	banana	5.0 ± 0.0	100	IV	+
Raman district, Yala, Thailand	RM1101	banana	5.0 ± 0.0	100	IV	+
	RM2101	banana	5.0 ± 0.0	100	IV	+
Department of Agriculture, Thailand	*Ralstonia solanacearum* 832	tomato	0.0 ± 0.0	0	I	–
	Ralstonia solanacearum 1350	ginger	0.0 ± 0.0	0	I	–
	Ralstonia solanacearum 1481	siam tulip	0.0 ± 0.0	0	I	–

DSS – disease severity score (0–5) on 'Hin' banana; DI% – disease incidence percentage on 'Hin' banana, Siam tulip: *Curcuma alismatifolia*

Two isolates, MY4101 and BT4101, were selected as representatives of the pathogen and their identities were determined based on their 16S rRNA gene sequences. A BLAST analysis of these sequences showed a 100% match with *R. syzygii* subsp. *celebesensis* (*Rsc*) (accession number KC757066.1). To further investigate their phylogenetic relationship, a Maximum Likelihood phylogenetic tree was constructed using the aligned 16S rRNA gene sequences. The tree revealed that isolates MY4101 and BT4101 clustered together in the same clade as *Rsc* (Fig. 1). This phylogenetic analysis provides strong evidence that these isolates belong to the same clade as *Rsc*, confirming their identification based on the 16S rRNA gene sequences.

Rsc-resistance banana cultivars

Of the 16 banana accessions tested in this study, three accessions of wild-type bananas, namely HB203, HB210, and HB247, exhibited an average disease severity score (DSS) ranging from 4.1 ± 1.2 to 4.6 ± 0.5. They showed a 100% disease incidence (DI), 85–90% disease index (DSI), and were classified as disease-susceptible (S) to *Rsc* isolates BT4101 and MY4101. Another wild-type accession, HB220, displayed an average DSS of 2.2 ± 0.5, 100% DI, 45% DSI, and was rated as moderately resistant (MR) to *Rsc* isolate BT4101 (Table 5).

Among the 12 accessions belonging to 11 banana cultivars, which were from three genome groups (AA, AAA, and ABB), the AA group accession BK002, known as 'Khai Kasetsart 2', was classified as resistant (R) to *Rsc* isolates BT4101 and MY4101. It had an average DSS of 0.1 ± 0.3 and 0.0 ± 0.0 when inoculated with *Rsc* isolates MY4101 and BT4101, respectively. The accession HB024, known as 'Raksa', was rated as MR to *Rsc* isolates BT4101 and MY4101(Table 5, Fig. 2).

Within the AAA group, five accessions including BK003 ('Nio Jorakhe'), HB227 ('Krang'), HB239 ('Hom Thong Pa'), HB243 ('Hom Thong'), and BK004 ('Hom Taiwan') were classified as susceptible (S) to both isolates of *Rsc*. Two accessions from the cultivar 'Hom Khiew Khom', HB132 and HB237, were rated as MR to *Rsc* isolate BT4101 but were classified as S to *Rsc* isolate MY4101. Accession HB240 ('Hom Africa') was rated as MR to *Rsc* isolate MY4101 but S to *Rsc* isolate BT4101 (Table 5, Fig. 2).

PR genes expression

The expression levels of the *PR3* and *PR10* genes were analyzed in two banana cultivars: 'Khai Kasetsart 2' (*Rsc*-resistant) and 'Hin' (*Rsc*-susceptible), on different days after inoculation (DAI) with *Rsc*.

PR3 gene expression showed significant differences between *Rsc*-inoculated 'Khai Kasetsart 2' and 'Hin'. In 'Khai Kasetsart 2', *PR3* gene expression was rapidly upregulated on 1 DAI and significantly higher than *Rsc*-inoculated 'Hin'. On 3 and 7 DAI, *PR3* gene expression in 'Khai Kasetsart 2' remained upregulated but was at lower levels than on 1 DAI. On the other hand, *Rsc*-inoculated 'Hin' showed lower *PR3* gene expression than 'Khai Kasetsart 2'. By 14 DAI, *PR3* gene expression was downregulated in 'Khai Kasetsart 2' compared to *Rsc*-inoculated 'Hin' and mock-inoculated bananas (Fig. 3A).

PR10 gene expression, *Rsc*-inoculated 'Khai Kasetsart 2' exhibited significant upregulation 1 DAI, which continued to increase 3 and 7 DAI. However, 14 DAI, *PR10* gene expression was significantly downregulated. Similarly, *Rsc*-inoculated 'Hin' showed higher *PR10* gene expression 7 DAI, but the expression was lower than 'Khai Kasetsart 2' (Fig. 3B).

Notably, the *PR10* gene in 'Khai Kasetsart 2' exhibited rapid upregulation from 1 to 7 DAI, while in the

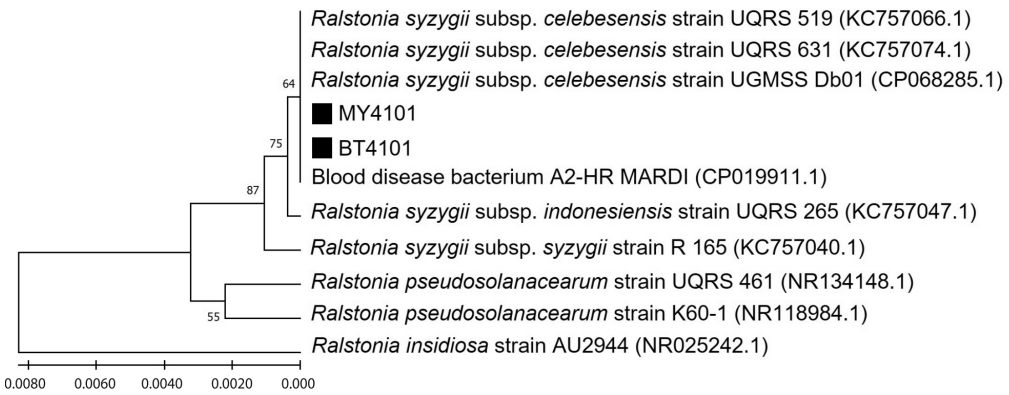

Fig. 1. Phylogenetic analysis of partial 16S rRNA gene sequences data using the Maximum Likelihood method based on the Tamura-Nei model. v indicates bacterial isolates from this study, including MY4101 (Accession Number: OR215006) and BT4101 (Accession Number: OR215005)

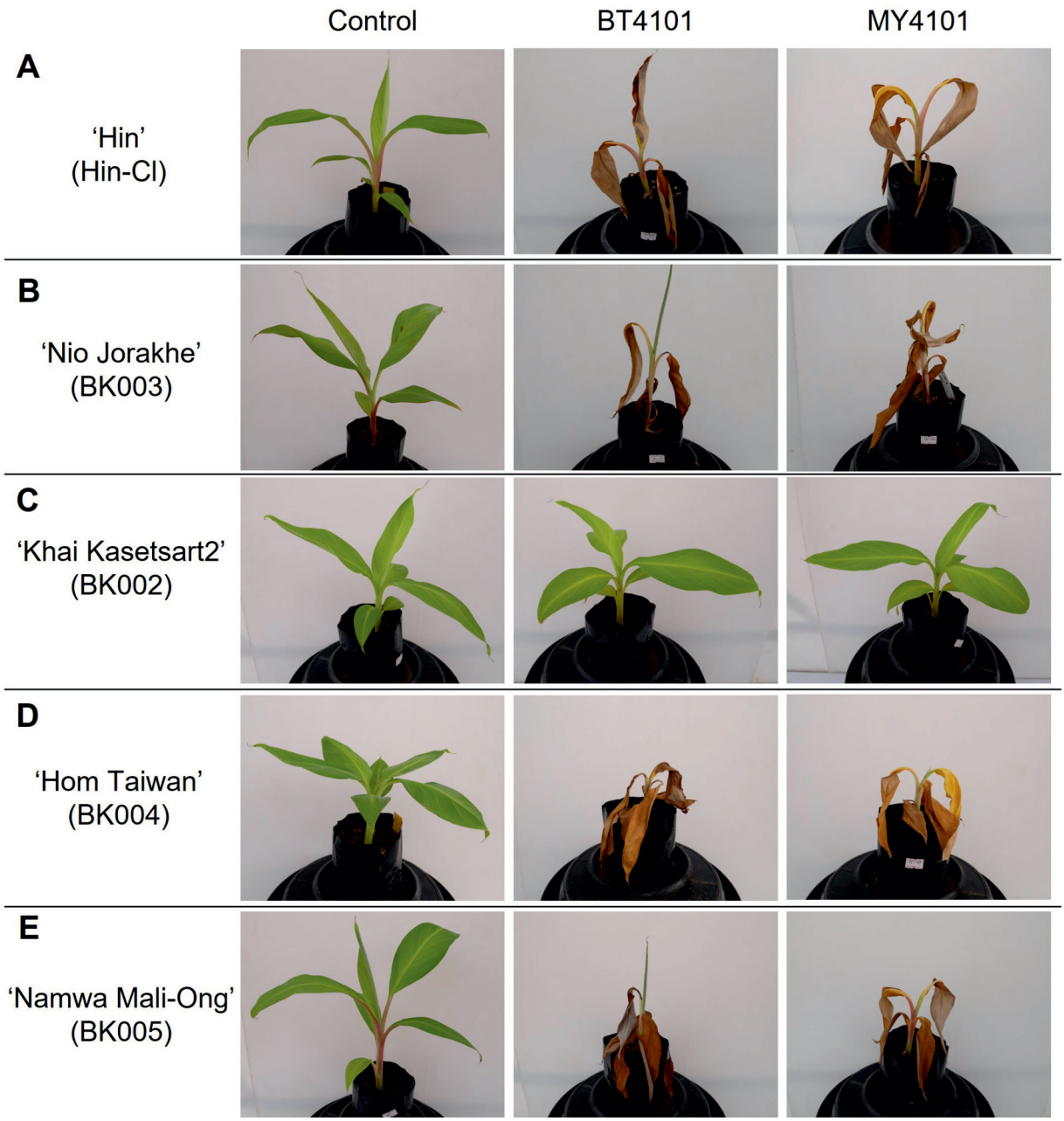

Fig. 2. Disease symptoms of some banana cultivars inoculated with *Rsc* isolate BT4101 and MY4101 4 weeks after inoculation. Control – mock inoculation

'Hin' cultivar, upregulation occurred 3 DAI followed by rapid downregulation. These expression patterns correlated with the disease severity score (DSS), disease index (DSI), and disease symptoms observed in the inoculated plants (Fig. 4 and Table 6).

Discussion

The bacteria causing banana blood disease (BBD) in banana plantations within Yala Province, Thailand, was confirmed through isolation and pathogenicity testing. The isolated bacteria were classified as *Ralstonia* spp. phylotype IV, which is the phylotype associated with the blood disease bacteria (Fegan and Prior 2006). BBD-specific PCR further confirmed the presence of *R. syzygii* subsp. *celebesensis* (*Rsc*) in the isolated samples (Das 2004). The representative isolates MY4101 and BT4101 were classified as members of the *Rsc* group based on their high similarity percentage (>98.65%, Kim and Chun 2014) in the 16S rRNA gene comparison, confirming their identification as *Rsc*.

Traditionally, screening for wilt disease-resistant banana cultivars, specifically against Fusarium wilt caused by *Fusarium oxysporum* f. sp. *cubense*, has been conducted through small-scale greenhouse studies in controlled environments (Zuo *et al.* 2018; Chen *et al.*

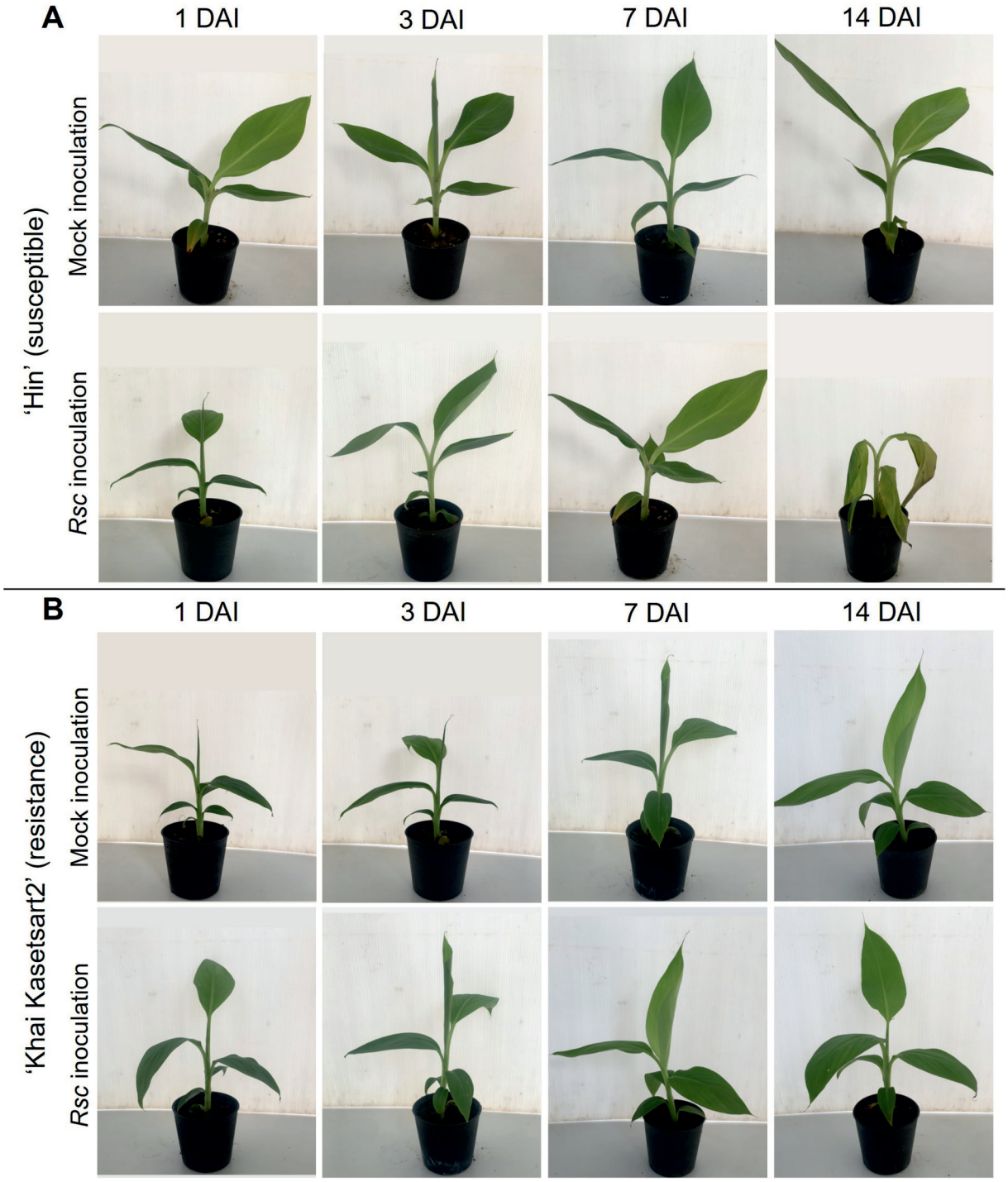

Fig. 3. Disease symptoms of 'Hin' (A) and 'Khai Kasetsart 2'(B) inoculated with *Rsc* isolate BT4101 1, 3, 7, and 14 days after inoculation

2019). To expand this screening approach, we assessed the resistance of banana cultivars to the bacterium *Rsc* in a greenhouse setting. By evaluating disease severity score (DSS), disease index (DSI), and disease resistance type (DRT), we observed variations among different banana cultivars. It is important to note that these scores were influenced by both the specific banana cultivar and the bacterial pathogen isolate.

Our evaluation of banana cultivars for resistance to *Rsc* revealed distinct responses among the tested accessions. The wild-type bananas HB203, HB210, and HB247 were classified as disease-susceptible (S) when exposed to *Rsc* isolates BT4101 and MY4101. However, the wild-type accession HB220 displayed moderate resistance (MR) to *Rsc* isolate BT4101. Among the cultivated banana accessions, the 'Khai Kasetsart 2' (BK002) cultivar from the AA genome group exhibited resistance (R) to both *Rsc* isolates, demonstrating significantly lower DSS than the susceptible 'Hin' cultivar. These findings highlight the high resistance

Table 5. Disease severity score, disease severity index, and disease resistance type of banana cultivars inoculated with *Rsc* isolate BT4101 and MY4101 4 weeks after inoculation

Cultivar (Group)	Accession	Rsc isolate BT4101				Rsc isolate MY4101			
		DSS	DI%	DSI%	DRT	DSS	DI%	DSI%	DRT
Wild type	HB203	4.5 ± 0.8	100	90	S	4.6 ± 0.5	100	93	S
	HB210	4.6 ± 0.5	100	93	S	4.6 ± 0.5	100	93	S
	HB220	2.2 ± 0.5	100	45	MR	4.1 ± 1.2	100	83	S
	HB247	4.5 ± 0.8	100	90	S	4.2 ± 0.9	100	85	S
'Khai Kasetsart 2'(AA)	BK002	0.0 ± 0.0	0	0	R	0.1 ± 0.3	10	3	R
'Raksa'(AA)	HB024	0.6 ± 1.2	20	13	MR	0.6 ± 1.2	20	13	MR
'Nio Jorakhe'(AAA)	BK003	4.6 ± 0.7	100	93	S	5.0 ± 0.0	100	100	HS
'Hom Khiew Khom' (AAA)	HB132	0.5 ± 0.5	40	10	MR	4.6 ± 0.5	100	93	S
	HB237	1.0 ± 0.0	100	20	MR	4.1 ± 0.8	100	83	S
'Krang' (AAA)	HB227	4.9 ± 0.3	100	98	S	4.5 ± 0.5	100	90	S
'Hom Thong Pa'(AAA)	HB239	2.6 ± 0.5	100	53	S	4.6 ± 0.5	100	93	S
'Hom Africa'(AAA)	HB240	4.4 ± 0.7	100	88	S	0.5 ± 0.8	30	10	MR
'Hom Thong'(AAA)	HB243	4.1 ± 0.8	100	83	S	4.7 ± 0.5	100	95	S
'Hom Taiwan'(AAA)	BK004	4.7 ± 0.5	100	95	S	4.7 ± 0.5	100	95	S
'Hin'(ABB)	Hin-CI	4.7 ± 0.5	100	95	S	5.0 ± 0.0	100	100	HS
'Namwa Mali-Ong' (ABB)	BK005	4.7 ± 0.5	100	95	S	4.7 ± 0.5	100	95	S

DSS – disease severity score (0–5); DI – disease incidence; DSI – disease index; DRT – disease resistance type defined by DSI (DSI ≤ 5% – R, resistance; DSI < 50% – MR, moderately resistance; DSI > 50% – S, susceptible; 100% DSI – HS, highly susceptible)

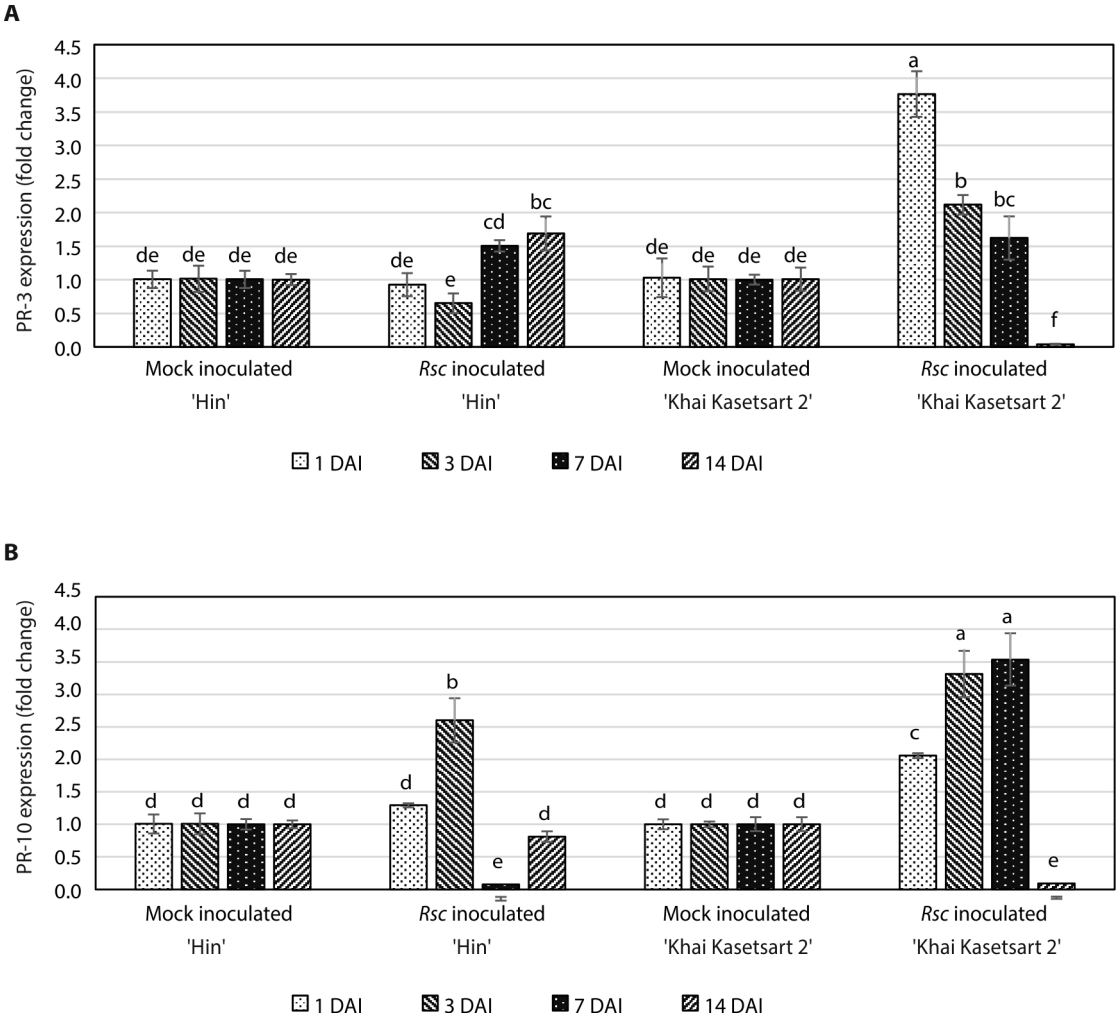

Fig. 4. *PR3* (A) and *PR10* (B) gene expression in 'Hin' and 'Khai Kasetsart 2' after *Rsc* inoculation

Table 6. Disease severity score and disease index of mock- and *Rsc*-inoculated 'Hin' and 'Khai Kasetsart 2' bananas on different days after inoculation

Treatments	1 DAI		3 DAI		7 DAI		14 DAI	
	DSS	DSI%	DSS	DSI%	DSS	DSI%	DSS	DSI%
'Hin' (susceptible)								
– Mock inoculation	0.0	0	0.0	0	0.0	0	0.0	0
– *Rsc* inoculation	0.0	0	0.0	0	1.0	16.7	3.6	60.0
'Khai Kasetsart 2' (resistance)								
– Mock inoculation	0.0	0	0.0	0	0.0	0	0.0	0
– *Rsc* inoculation	0.0	0	0.0	0	0.0	0	0.0	0

DSS – disease severity score (0–5); DSI – disease index; DAI – day after inoculation

levels of the 'Khai Kasetsart 2' cultivar to *Rsc*, making it a promising candidate for future conventional breeding programs. Moreover, 'Khai Kasetsart 2' can be a resistant control cultivar for improving susceptible bananas through mutagenesis or genetic modification. However, considering the potential influence of different environmental conditions, field trials should be conducted to assess the performance of this resistant cultivar over an extended period before its widespread implementation (Mintoff *et al.* 2021).

In order to elucidate the molecular mechanisms underlying defense responses in the 'Khai Kasetsart 2' and 'Hin' cultivars, we analyzed the expression levels of *PR3* and *PR10* genes following *Rsc* inoculation. We found that *PR3* gene expression was significantly upregulated in 'Khai Kasetsart 2' on day 1 after inoculation (DAI) compared to *Rsc*-inoculated 'Hin'. This suggests an early and sustained upregulation of the *PR3* gene in the *Rsc*-resistant 'Khai Kasetsart 2' cultivar, potentially contributing to its enhanced resistance against *Rsc*. The *PR3* gene family encodes class II chitinases, which are crucial in regulating plant resistance and preventing pathogen infections (Tang *et al.* 2017). Tang *et al.* (2017) demonstrated that overexpressing *NtPR-Q*, a member of the *PR3* family encoding endochitinases, led to the upregulation of defense-related genes and enhanced resistance to *R. solanacearum* infection through salicylic acid (SA), jasmonic acid (JA), and ethylene (ET)-mediated defense signaling pathways.

In *PR10* gene expression, *Rsc*-inoculated 'Khai Kasetsart 2' exhibited significant upregulation 1 DAI, further increasing 3 and 7 DAI. However, 14 DAI, *PR10* gene expression was significantly downregulated. The 'Hin' cultivar showed higher *PR10* gene expression 7 DAI which was lower than 'Khai Kasetsart 2'. These expression patterns suggest a dynamic regulation of the *PR10* gene in response to *Rsc* infection. The rapid upregulation of *PR10* gene expression in 'Khai Kasetsart 2' during the early stages of infection indicates its potential role in the defense response. However, the subsequent downregulation 14 DAI suggests the activation of other regulatory mechanisms or the completion of the defense response. *PR-10* proteins (ribonuclease-like) belong to the intracellular PR (IPR) protein group and are small multifunctional proteins involved in plant defense responses against various biotic stresses, including fungal, viral, and bacterial pathogens, as well as abiotic stresses (Jain and Kumar 2015; Finkina *et al.* 2017). Choi *et al.* (2012) demonstrated that suppressing cytosolic *PR-10*/*LRR1* in transgenic peppers led to the restriction of avirulent *Xanthomonas campestris* pv. *vesicatoria* infection through cell death-mediated defense signaling. These findings further support the role of *PR3* and *PR10* proteins in preventing pathogen colonization at the infection site.

In conclusion, this study provides valuable insights into the pathogenicity of *Rsc* isolates causing banana blood disease (BBD) and the resistance responses of different banana cultivars. The isolated bacteria were identified as *R. syzygii* subsp. *celebesensis* (*Rsc*), belonging to phylotype IV. The 'Khai Kasetsart 2' cultivar resisted *Rsc* and showed distinct expression patterns of *PR3* and *PR10* genes compared to the susceptible 'Hin' cultivar. These findings contribute to our understanding of the molecular mechanisms underlying *Rsc* resistance in bananas and potentially offer future strategies for breeding and managing BBD-resistant cultivars. In the future, focusing on the specific defense mechanisms activated in resistant cultivars is warranted in order to unravel the complex interactions between *Rsc* and banana plants.

Acknowledgements

This research was funded by Kasetsart University through the Graduate School Fellowship Program and the Agricultural Research Development Agency (Public Organization) of Thailand or "ARDA". The authors would like to express their gratitude to the Faculty of Natural Resources and Agro-Industry, Kasetsart

University Chalermphrakiat Sakon Nakhon Province Campus, for their support in facilitating this project. Special thanks are extended to Dr. Araya Arjcharoen Theanhom and Monthar Wongmaneeroj for providing the clean cultures of banana cultivars.

References

Andersen E.J., Ali S., Byamukama E., Yen Y., Nepal M.P. 2018. Disease resistance mechanisms in plants. Genes 9: 339. DOI: https://doi.org/10.3390/genes9070339

Bakar R.A.H., Badrun R., Ahmad K., Bakar N.A. 2018. Symptomatology and range of the blood disease bacterium A2 HR MARDI strain (*Ralstonia syzygii* subsp. *celebesensis*) on selected hosts. IOSR Journal of Agriculture and Veterinary Science (IOSR-JAVS) 11: 71–77.

Blomme G., Dita M., Jacobsen K.S., Pérez Vicente L., Molina A., Ocimati W., Poussier S., Prior P. 2017. Bacterial diseases of bananas and enset: current state of knowledge and integrated approaches toward sustainable management. Frontiers in Plant Science 8: 1290. DOI: https://doi.org/10.3389/fpls.2017.01290

Buddenhagen I. 2009. Blood bacterial wilt of banana: history, field biology and solution. Acta Horticulturae 828: 57–68. DOI: https://doi.org/10.17660/ActaHortic.2009.828.4

Chen A., Sun J., Matthews A., Armas-Egas L., Chen N., Hamill S., Mintoff S., Tran-Nguyen L.T., Batley J., Aitken E.A. 2019. Assessing variations in host resistance to *Fusarium oxysporum* f sp. *cubense* race 4 in *Musa* species, with a focus on the subtropical race 4. Frontiers in Microbiology 10: 1062. DOI: https://doi.org/10.3389/fmicb.2019.01062

Choi D.S., Hwang I.S., Hwang B.K. 2012. Requirement of the cytosolic interaction between pathogenesis-related protein 10 and leucine-rich repeat protein 1 for cell death and defense signaling in pepper. The Plant Cell 24: 1675–1690. DOI: https://doi.org/10.1105/tpc.112.095869

Dangl J.L., Horvath D.M., Staskawicz B.J. 2013. Pivoting the plant immune system from dissection to deployment. Science 341: 746–751. DOI: https://www.science.org/doi/10.1126/science.1236011

Das S.H.J. 2004. Molecular diagnostics of the Blood Disease Bacterium. Doctoral dissertation, Honours thesis. The University of Queensland, Brisbane, QLD, Australia.

Davis R.I., Moore N.Y., Fegan M. 2001. Blood disease and panama disease: two newly introduced and grave threats to banana production on the island of New Guinea. p. 816–821. In: "Food Security for Papua New Guinea" (R.M. Bourke, M.G. Allen, J.G. Salisbury, eds.). ACIAR proceedings, No. 99.

Decreux A., Messiaen J. 2005. Wall-associated kinase WAK1 interacts with cell wall pectins in a calcium-induced conformation. Plant and Cell Physiology 46: 268–278. DOI: https://doi.org/10.1093/pcp/pci026

Ebrahim S., Usha K., Singh B. 2011. Pathogenesis related (PR) proteins in plant defense mechanism. p. 1043–1054. In: "Science Against Microbial Pathogens: Communicating Current Research and Technological Advances" (A. Méndez-Vilas, ed.). FORMATEX, Spain.

Fegan M., Prior P. 2005. How complex is the *Ralstonia solanacearum* species complex. p. 449–461. In: "Bacterial Wilt Disease and the *Ralstonia solanacearum* Species Complex" (C. Allen, P. Prior, A. C. Hayward, eds). APS Press, St. Paul, MN, USA.

Fegan M. Prior P., 2006. Diverse members of the *Ralstonia solanacearum* species complex cause bacterial wilts of banana. Australasian Plant Pathology 35: 93–101. DOI: https://doi.org/10.1071/AP05105

Finkina E.I., Melnikova D.N., Bogdanov I.V., Ovchinnikova T.V. 2017. Plant pathogenesis-related proteins PR-10 and PR-14 as components of innate immunity system and ubiquitous allergens. Current Medicinal Chemistry 24: 1772–1787. DOI: https://doi.org/10.2174/0929867323666161026154111

Jain S., Kumar A. 2015. The pathogenesis related class 10 proteins in plant defense against biotic and abiotic stresses. Advances in Plants & Agriculture Research 3: 00077. DOI: https://doi.org/10.15406/apar.2015.02.00077

Kelman A. 1954. The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. Phytopathology 44: 693–695.

Kim M., Chun J. 2014. 16S rRNA gene-based identification of bacteria and archaea using the EzTaxon server. Methods in Microbiology 41: 61–74. DOI: https://doi.org/10.1016/bs.mim.2014.08.001

Lane D.J. 1991. 16S/23S rRNA sequencing. p. 115–175. In: "Nucleic acid Techniques in Bacterial Systematics" (E. Stackebrandt, M. Goodfellow, eds.). John Wiley & Sons, New York, USA.

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

Mintoff S.J., Nguyen T.V., Kelly C., Cullen S., Hearnden M., Williams R., Daniells J.W., Tran-Nguyen L.T. 2021. Banana cultivar field screening for resistance to *Fusarium oxysporum* f. sp. *cubense* tropical race 4 in the northern territory. Journal of Fungi 7: 627. DOI: https://doi.org/10.3390/jof7080627

Murashige T., Skoog F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum 15: 473–497. DOI: https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Opina N., Tavner F., Hollway G., Wang J.F., Li T.H., Maghirang R., Fegan M., Hayward A.C., Krishnapillai V., Hong W.F., Holloway B.W., Timmis J. 1997. A novel method for development of species strain-specific DNA probes and PCR primers for identifying *Burkholderia solanacearum* (formerly *Pseudomonas solanacearum*). Asia-Pacific Journal of Molecular Biology and Biotechnology 5: 19–30.

Osmond R.I., Hrmova M., Fontaine F., Imberty A., Fincher G.B. 2001. Binding interactions between barley thaumatin-like proteins and (1, 3)-β-D-glucans: Kinetics, specificity, structural analysis and biological implications. European Journal of Biochemistry 268: 4190–4199. DOI: https://doi.org/10.1046/j.1432-1327.2001.02331.x

Safni I., Cleenwerck I., De Vos P., Fegan M., Sly L., Kappler U. 2014. Polyphasic taxonomic revision of the *Ralstonia solanacearum* species complex: proposal to emend the descriptions of *Ralstonia solanacearum* and *Ralstonia syzygii* and reclassify current *R. syzygii* strains as *Ralstonia syzygii* subsp. *syzygii* subsp. nov., *R. solanacearum* phylotype IV strains as *Ralstonia syzygii* subsp. *indonesiensis* subsp. nov., banana blood disease bacterium strains as *Ralstonia syzygii subsp. celebesensis* subsp. nov. and *R. solanacearum* phylotype I and III strains as *Ralstonia pseudosolanacearum* sp. nov. International Journal of Systematic and Evolutionary Microbiology 64: 3087–3103. DOI: https://doi.org/10.1099/ijs.0.066712-0

Simmonds N.W., Shepherd K. 1955. The taxonomy and origins of the cultivated bananas. Biological Journal of the Linnean Society 55: 302–312. DOI: https://doi.org/10.1111/j.1095-8339.1955.tb00015.x

Sinha M., Singh R.P., Kushwaha G.S., Iqbal N., Singh A., Kaushik S., Kaur P., Sharma S., Singh T.P. 2014. Current overview of allergens of plant pathogenesis related protein families. The Scientific World Journal 2014: Article ID 543195. DOI: https://www.hindawi.com/journals/tswj/2014/543195/

Supriadi. 2005. Present status of blood disease in Indonesia. p. 395–404. In: "Bacterial Wilt Disease and the *Ralstonia solanacearum* Species Complex" (C. Allen, P. Prior, A.C. Hayward, eds.). American Phytopathological Society (APS Press), USA.

Tang Y, Liu Q, Liu Y, Zhang L, Ding W. 2017. Overexpression of NtPR-Q up-regulates multiple defense-related genes in *Nicotiana tabacum* and enhances plant resistance to *Ralstonia solanacearum*. Frontiers in Plant Science 8. DOI: https://doi.org/10.3389/fpls.2017.01963

Teng S.K., Aziz N.A., Mustafa M., Laboh R., Ismail I.S., Sulaiman S.R., Ain Azizan A., Devi S. 2016. The occurrence of blood disease of banana in Selangor, Malaysia. International Journal of Agriculture and Biology 18: 92–97. DOI: https://doi.org/10.17957/IJAB/15.0067

van Loon L.C. 1999. Occurrence and properties of plant pathogenesis-related proteins. p. 1–19. In: "Pathogenesis-Related Proteins in Plants" (S.K. Datta, S. Muthukrishnan, eds.). CRC Press LLC.

Zipfel C. 2014. Plant pattern-recognition receptors. Trends in Immunology 35: 345–351. DOI: https://doi.org/10.1016/j.it.2014.05.004

Zuo C., Deng G., Li B., Huo H., Li C., Hu C., Kuang R., Yang Q., Dong T., Sheng O.U., Yi G. 2018. Germplasm screening of *Musa* spp. for resistance to *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (*Foc* TR4). European Journal of Plant Pathology 151: 723–734. DOI: https://doi.org/10.1007/s10658-017-1406-3