Jurnal Perlindungan Tanaman Indonesia, Vol. 27, No. 2, 2023: 86–92
DOI: 10.22146/jpti.81099
Available online at http://jurnal.ugm.ac.id/jpti
ISSN 1410-1637 (print), ISSN 2548-4788 (online)

Research Article

Primer Design of Volatile Synthesis Coding Genes in *Ralstonia syzygii* subsp. *celebesensis*

Nina Septania Damanik[1)], Ady Bayu Prakoso[1)], Kuwat Triyana[2)], & Siti Subandiyah[1)]*

[1)]*Department of Plant Protection, Faculty of Agriculture, Universitas Gadjah Mada*
Jln. Flora No. 1, Bulaksumur, Sleman, Yogyakarta 55281 Indonesia
[2)]*Department of Physics, Faculty of Mathematics and Natural Sciences, Universitas Gadjah Mada*
Sekip Utara, BLS 21, Yogyakarta 55281, Indonesia

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

Received January 16, 2023; revised May 8, 2023; accepted December 7, 2023

ABSTRACT

Microbes produce various types of volatile organic compounds (VOCs) through metabolism, which can be used for diagnostic purposes. Microbes' types and classes of VOCs are very wide, including fatty acid derivatives (hydrocarbons, alcohols, and ketones), aromatic compounds, nitrogen-containing compounds, and volatile sulfur compounds. Microbial volatile organic compounds (VOCs) can also be divided into several chemical classes: alkenes, alcohols, ketones, benzos, pyrazines, sulfides, acids, esters, and terpenes. This study aimed to design primers for genes encoding volatile synthesis in *Ralstonia syzygii* subsp. *celebesensis*, which causes blood disease in the banana plant. Some of the genes involved are *adc* (acetone synthesis), *adh*P (ethanol synthesis), *ilvA*, *nirBD* (ammonia synthesis), *mdcA* (propionic acid synthesis), *cysI* (hydrogen sulfide synthesis), and *speBC* (putrescine synthesis). Primers were designed and examined for specificity in silico using Primer3Plus, Geneious Prime, and BLAST programs. The numbers of nine pairs designed primers were successfully amplifying the related nine VOC genes of *R. syzygii* subsp. *celebesensis* for qPCR.

Keywords: *Ralstonia syzygii* subsp. *celebesensis*; PCR; primers; VOC

INTRODUCTION

Ralstonia syzygii subsp. *celebesensis* is a gram-negative bacterium pathogenic to banana plants that causes the blood disease bacterium (BDB). It belongs to phylotype IV of the *Ralstonia solanacearum* species complex (Fegan & Prior, 2005; Safni *et al.*, 2014). BDB is one of the important banana wilt diseases, which can cause damage to banana plantations of up to 27–80%, causing significant crop losses in Indonesia and Malaysia (Blomme *et al.*, 2017; Ray *et al.,* 2021). BDB has been known to attack banana plants in Indonesia since 1921 (Remenant *et al.,* 2011). Blood disease in banana plants was originally only found in Indonesia, but now this disease has spread in Selangor, Malaysia, which is associated with Moko and Fusarium wilt disease (Teng *et al.,* 2016).

Diagnostic methods are an important component in disease management on banana plants that are vegetatively propagated to prevent the spread of diseases (Rincon-Florez *et al.,* 2021). Besides that, fast and accurate early detection is needed to prevent the spread and wider outbreak of BDB. It is difficult to detect asymptomatic blood diseases. Currently, methods for detecting BDB such as serological methods, *loop-mediated isothermal amplification* methods, conventional PCR, and qPCR are available (Baharuddin *et al.,* 1994; Edy *et al.,* 2011; Kubota *et al.,* 2011; Lenarčič *et al.,* 2014; EPPO, 2018; Rincon-Florez *et al.,* 2021; Talib *et al.,* 2021). Although these methods have proven reliable, they are relatively difficult to operate, require expensive materials and skilled technicians, and take a long time to analyze data (Cui *et al.,* 2018). These methods cannot provide real-time detection, making them less suitable for field testing and early warning systems.

Various methods or technologies have been developed, which can be divided into two approaches:

direct and indirect. Direct detection approaches include molecular technologies and serological technologies such as enzyme-linked immunosorbent assay (ELISA). Meanwhile, the typical indirect method detects morphological changes, changes in transpiration rate, and volatile organic compound (VOC) profiles, which are in accordance with fluorescence imaging technology, hyperspectral techniques, and gas chromatography-mass spectrometry (GC-MS) (Cui *et al.,* 2018).

It is well-known that both microbes and plants emit volatile compounds, some of which may have odors that are characteristic of the species (Blasioli, 2014). Bacteria can produce various types of volatile compounds. Bacterial volatile compounds are known to contribute to interkingdom interactions (plants, fungi, and nematodes), and are also involved in antagonism, mutualism, and internal and inter-species regulation of cellular and growth metabolisms (Bitas *et al.,* 2013; Audrain *et al.,* 2015). The volatile compounds produced by the *Ralstonia solanacearum* species complex were tested on *Ralstonia solanacearum* in the other study. The volatile compounds produced were chemical compounds such as alcohols, aldehydes, alkanes, alkyls, amides, amines, benzene, ketones, siloxanes, and thiols (Spraker *et al.,* 2014).

We explored in silico the genes involved in the synthesis of volatile compounds in *R. syzygii* subsp. *celebesensis*. Several genes are involved, such as *adc* (acetone), *adh*P (ethanol), *ilvA*, *nirBD* (ammonia), *mdcA* (propionic acid), *cysI* (hydrogen sulfide), and *speBC* (putrescine). Here, we demonstrate the existence of volatile encoding genes in *R. syzygii* subsp. *celebesensis*. management.

MATERIALS AND METHODS

Primer Design

This study used the *R. syzygii* subsp. *celebesensis* UGMSS_Db01 strain, whose genome was sequenced and submitted to GenBank and assigned accession numbers NZ_CP068285 for chromosomes and NZ_CP068286 for plasmids (Prakoso, 2022). PCR primers were designed to amplify volatile synthesis-encoding genes. The selected encoding genes are those involved in the synthesis of acetone, alcohol, hydrogen sulfide, ammonia, propanoic acid, and putrescine. The candidate gene sequences were taken from gene sequences at the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/). Gene sequences in nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to find out their homologs in NCBI. Then the candidate sequences from the BLAST results were selected that had the highest similarity to *R. syzygii* subsp. *celebesensis* UGMSS_Db01. Primer candidates were analyzed using NetPrimer-Biosoft software to check primer pairs' quality and Tm values. The sequence is then BLAST the protein to find out the results of the protein as translated by the gene. The resulting sequences of candidate genes were selected for their primer pairs using the Primer3Plus (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi) and Geneious Prime software.

The primer candidates were then BLAST to confirm that the gene came from *R. syzygii* subsp. *celebesensis* UGMSS_Db01. Then the candidate primer pairs were performed in-silico PCR, or primers were attached to *R. syzygii* subsp. *celebesensis* sequences using the SnapGene Viewer software to ensure that the primers were suitable for *R. syzygii* subsp. *celebesensis*.

DNA extraction and PCR conditions

The strain *R. syzygii* subsp. *celebesensis* UGMSS_Db01 was streaked onto Casein Peptone Glucose (CPG) agar plates 1 g casein, 10 g peptone, and 5 g glucose per liter and incubated at 30°C for 48−72 hours before further preparation. Total DNA extraction was performed using the Presto™ Mini gDNA Bacteria Kit (Geneaid) according to the manufacturer's instructions for Gram-negative bacteria. All gDNA samples were stored at −20°C until used as templates in the PCR assays. All primers used in this study to determine the existence of genes encoding for volatile synthesis are listed in Table 1. *R. syzygii* subsp. *celebesensis* were also amplified using specific primers 121F (5'-CGT ATT GGA TGC CGT AAT GGA-3') and 121R (5'-AAG TTC ATT GGT GCC GAA TCA-3'). For each gene, PCR amplification was performed in 10 µL of the total volume, consisting of 5 µl of ReadyMix, 1 µl of F primer and R primer (10 pmol), 2 µl of nuclease-free water, and 1µl of gDNA. DNA fragment application was performed using a PCR machine. After an initial denaturation at 95°C for 3 min,

Table 1. *Ralstonia syzygii* subsp. *celebesensis* UGMSS_Db01 strain primers designed for qPC

Genes	Gene ID	Primers Sequence 5' → 3'		start	length	GC (%)	ΔTm	Amplicon (bp)
adc	JK151_21925	F	GTGCTGGAAATCGTGGAGGC	658	20	60	0.4	116
		R	AAGGGTGGGATGGAACGGAC	773	20	60		
adhP	JK151_03205	F	GCTATGCCGAATACGTGCTG	368	20	55	0.56	121
		R	ATGCGGATGCCCTTGTAGAC	488	20	55		
ilyA	JK151_00610	F	AAGAAGATCCTGACCGCCAA	19	20	50	0.5	140
		R	CGCAGCTTGAACGAGAACAC	158	20	55		
nirB	JK151_06690	F	CGACCGCTTCCTGATGTTCT	2119	20	55	0.1	112
		R	GCCCACGATGACTTCCTTCA	2310	20	55		
nirD	JK151_06685	F	GACATCGTACCCAACACCGG	40	20	60	0.6	119
		R	GAGTTCGGGTCGTAGTTGCC	158	20	57.9		
speB	JK151_18620	F	ATTACTACATCACCATCGACGC	748	22	45.5	0.6	118
		R	ACCAGGCCGTGAATTAGCT	865	19	52.6		
speC	JK151_17220	F	GAAGAGGGCATGGAGGTCAC	97	20	60	0.2	131
		R	TTGTCGTTGTCCGGGTTGAT	227	20	50		
cysI	JK151_17495	F	AAGAGGAGTGGCAGCACATC	803	20	55	0.1	109
		R	GTATCGGGCAGCTTGTCGTA	911	20	55		
mdcA	JK151_00505	F	GGCGGCTTCATCAACATCAC	1258	20	55	0.9	136
		R	GCACGATCTTCTTCAGGCG	1393	20	57.9		

samples were thermocycling for 35 cycles in the BioRad T100TM Thermal Cycle at 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min. The amplification results were visualized using gel electrophoresis. 1 µL of the amplification obtained was injected into 1% agarose and electrophoresed using an electrophoresis set (Bio-Rad) at 50 V for 50 minutes. The visualization results in the formation of thick bands ranging in size from 100 to 150 bp.

RESULTS AND DISCUSSION

Selection of Primers for the Volatile Synthesis-Encoding Gene

According to the initial primer design results from Primer3Plus, 10 forward primers and 10 reverse primers (10 primer sets) were obtained for each gene, as shown in Table 1. The primer pairs were then selected based on the length of the amplicon (100−150 bp), as the primers would be used for quantitative PCR. Furthermore, primers were chosen based on melting temperature (Tm) (42−65 °C) with a Tm difference of less than 1 °C, guanine or cytosine content (40−60%), the optimal length of primers (usually between 18 and 30 bases), the presence of G or C ends, the absence of repeating sequences with a maximum number of repeated sequences, the absence of run sequences or a maximum of 3 nucleotides, and no self-complementarity (Arenas & Salazar, 2019).

The products of bacterial metabolism with low vapor pressure, high lipophilicity, and a molecular weight of less than 300 Da are likely to be released as volatile compounds. The volatiles produced by bacteria reflects the specific metabolism of the bacteria. Given the enormous diversity of bacterial metabolism, these volatile compounds can originate from a variety of metabolic pathways (Schulz & Dickschat, 2007). Volatile profiles have been widely used in the identification of plant pathogenic bacteria. In this research, the primer design of the genes involved in the synthesis of volatiles was carried out, namely the genes encoding the synthesis of acetone, alcohol, ammonia, putrescine, hydrogen sulfide, and propanoic acid.

The gene involved in acetone synthesis investigated in this study is the *adc* gene, which has the function of producing the enzyme acetoacetate decarboxylase, which converts acetoacetate to acetone. Acetone is naturally present in the metabolism of plants, animals, and bacteria. The standard dogma has persisted for so long that acetone is the end product of metabolism in animals and humans. Acetone evaporates easily, even from water and soil (Kalapos, 2014). The addition of acetate and glucose to the bacterial growth substrate can increase the production of acetone (Gao *et al.,* 2015). The medium used in this study was CPG, which consisted

of 5 g/L glucose. To obtain energy, bacteria consume glucose via the pyruvic acid metabolism pathway; pyruvic acid is then converted into the compound Acetyl CoA. Acetyl CoA is converted into acetoacetate. Acetoacetate decarboxylase releases CO2 from acetoacetate to form acetone (Berzin *et al.,* 2012). The *adc* gene *R. syzygii* subsp. *celebesensis* is located at the JK151_21925 gene locus, which is involved in butanoate or butyric acid metabolism (KEGG, 2021).

The adh gene is a gene coding for this alcohol dehydrogenase enzyme (Atika *et al.,* 2015). On *R. syzygii* subsp. *celebesensis*, the alcohol dehydrogenase enzyme is encoded by *adh*P, which is located at the JK151_03205 gene locus. The enzyme alcohol dehydrogenase converts acetaldehyde to ethanol. Ethanol is produced from glucose through the consumption of pyruvate during fermentation (Kang, 2015). Glycolysis is a metabolic process that converts glucose into the oxidized product, pyruvate, and supplies ATP for the production of bacterial biomass. Furthermore, under anaerobic conditions, pyruvate can be fermented into ethanol through the sequential reactions of pyruvate decarboxylase and alcohol dehydrogenase, which lose one carbon as carbon dioxide (CO_2) (Kang, 2015).

The genes encoding for ammonia synthesis investigated in this study were the *ilvA*, *nirB*, and *nirD* genes. The *ilvA* gene catabolizes the amino acid threonine to -ketobutyrate, 2- isobutyrate, and ammonia. The *nirB* and *nirD* genes, which encode the enzyme nitrite reductase, are also involved in the formation of ammonia by converting nitrite compounds into ammonia. On *R. syzygii* subsp. *celebesensis*, the *ilvA* gene is located at the JK151_00610, the *nirB* gene is located at the JK151_RS06690, and the *nirD* gene is located at the JK151_RS06685 gene locus.

Hydrogen sulfide gas is produced during the process of sulfur metabolism by bacteria. Microorganisms can involve sulfur in their metabolism, both in oxidized and reduced states (Fauque & Barton, 2012; Kushkevych *et al.,* 2020). Sulfate reductase performs a major role in sulfur metabolism by catalyzing the reduction of sulfite to hydrogen sulfide and water (Erva *et al.,* 2018). In *E. coli*, the genes involved are encoded by the *cysJIH* operon, which consists of the gene for the sulfate reductase subunit (Kushkevych *et al.,* 2020). On *R. syzygii* subsp. *celebesensis*, sulfate reductase is encoded by the *cysI* gene (JK151_17495) (KEGG, 2021).

The *mdcA* gene is involved in the synthesis of propanoic acid. *R. syzygii* subsp. *celebesensis* has a propionate-CoA transferase at the JK151_00505 gene locus, which is involved in propanoate metabolism (KEGG, 2021). Putrescine is a putrid-smelling organic chemical compound produced by the breakdown of amino acids in living and dead organisms. Putrescin is largely responsible for the stench of rotting flesh (Boyle *et al.,* 1984). In *R. solanacearum*, an abundance of putrescine compounds was found as a virulence metabolite produced by the pathogen (Lowe-Power *et al.,* 2018). The putrescine compounds were formed through the action of the enzymes ornithine decarboxylase (*speC*) from ornithine compounds and agmatinase (*speB*) from arginine compounds. On *R. syzygii* subsp. *celebesensis*, enzymes involved in putrescine synthesis are located at the JK151_18620 agmatinase (*speB*) and JK151_17220 ornithine decarboxylase (*speC*) gene loci.

The Existence of Volatile Synthesis Coding Genes in *Ralstonia syzygii* subsp. *celebesensis*

The extraction of total genomic DNA was amplified using the PCR method. Primer 121F/R was used to prove that the extraction gene is *R. syzygii* subsp. *celebesensis*. The results of polymerization with primer 121FR showed that the DNA extract used was positive for RSC, indicated by the formation of DNA fragments with a size of 317 bp. The primer used for the detection of genes encoding volatile synthesis was also well amplified, as shown by the formation of DNA fragments with a size of 100−150 bp (Figure 1). The DNA base sequence specifications of primers 121F/R are able to recognize *R. syzygii* subsp. *celebesensis* in their total genomic DNA. The special characterization belonging to *R. syzygii* subsp. *celebesensis* on its DNA fragments will be the main identity factor as well as a distinguishing character from other bacteria (Edy *et al.,* 2011). The annealing temperature used in PCR was 60°C for the 30s, the same annealing temperature used in quantitative PCR (qPCR) for volatile gene expression analysis. The amplicons produced were also

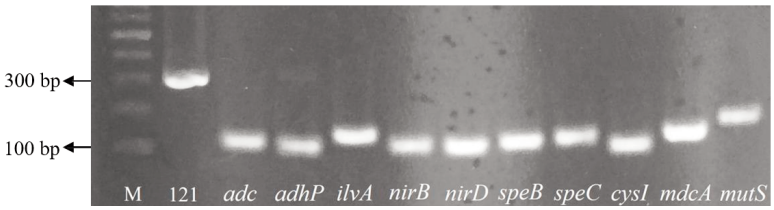

Figure 1. Amplification of *R. syzygii* subsp. *celebesensis* genomic DNA on PCR using primer 121F/R and primer for the gene encoding volatile synthesis showed that all primers could amplify the target gene

adjusted for the use of qPCR, which is 100−150 bp. In qPCR, the amplicon size directly influences the detection of the target: the larger the amplicons, the longer the detection (Debode *et al.,* 2017).

CONCLUSION

Based on this study, the *adc, adhP, ilvA, nirBD, cysI, mdcA,* and *speBC* genes are present in the genome of *R. syzygii* subsp. *celebesensis*. The pairs of primers designed in this study have the potential to be used as validations for the presence of volatile organic compounds (VOC) produced by *R. syzygii* subsp. *celebesensis*.

ACKNOWLEDGEMENT

The author would like to thank the Agency for Agricultural Extension and Human Resources Development (BPPSDMP), Ministry of Agriculture of the Republic of Indonesia for providing Master Scholarship and Research Funding from the BIMA Research Grant with contract number 2688/UN1/DITLIT/Dit-Lit/PT.01.03/2022. This article is part of the first author's Master thesis.

LITERATURE CITED

Arenas, N.E., & Salazar, L.M. (2019). Steps and Tools for PCR-Based Technique Design, In Lopes, E.J. & Zepka, L.Q. (Eds.), *Biotechnology and Bioengineering*. London: IntechOpen. https://doi.org/10.5772/intechopen.83671

Atika, Y., Artika, I.M., & Nurhidayat, N. (2015). Identifikasi Gen Termoasidofilik Alkohol Dehidrogenase pada *Bacillus* sp. *Berita Biologi*, *14*(1), 105−110.

Audrain, B., Farag, M.A., Ryu, C.M., & Ghigo, J.M. (2015). Role of Bacterial Volatile Compounds in Bacterial Biology. *FEMS Microbiology Reviews*, *39*(2), 222−233. https://doi.org/10.1093/femsre/fuu013

Baharuddin, B., Rudolph, K., & Niepold, F. (1994). Production of Monospecific Antiserum against the Blood Disease Bacterium Affecting Banana and Plantain. *Phytopathology*, *84*(6), 570−575. https://doi.org/10.1094/Phyto-84-570

Berzin, V., Kiriukhin, M., & Tyurin, M. (2012). Selective Production of Acetone during Continuous Synthesis Gas Fermentation by Engineered Biocatalyst *Clostridium* sp. MAceT113. *Letters in Applied Microbiology*, *55*(2), 149–154. https://doi.org/10.1111/j.1472-765X.2012.03272.x

Bitas, V., Kim, H.S., Bennett, J.W., & Kang, S. (2013). Sniffing on Microbes: Diverse Roles of Microbial Volatile Organic Compounds in Plant Health. *Molecular Plant-Microbe Interactions, 26*(8), 835-843. http://doi.org/10.1094/MPMI-10-12-0249-CR

Blasioli, S., Biondi, E., Samudrala, D., Spinelli, F., Cellini, A., Bertaccini, A., Cristescu, S.M., & Braschi, I. (2014). Identification of Volatile Markers in Potato Brown Rot and Ring Rot by Combined GC-MS and PTR-MS Techniques: Study on In Vitro and In Vivo Samples. *Journal of Agricultural and Food Chemistry*, *62*(2), 337–347. https://doi.org/10.1021/jf403436t

Blomme, G., Dita, M., Jacobsen, K.S., Vicente, L.P., Molina, A., Ocimati, W., Poussier, S., & Prior, P. (2017). Bacterial Diseases of Bananas and Enset: Current State of Knowledge and Integrated Approaches towards Sustainable Management. *Frontiers in Plant Science, 8*(1290), 1–25. https://doi.org/10.3389/fpls.2017.01290

Boyle, S.M., Markham, G.D., Hafner, E.W., Wright, J.M., Tabor, H., & Tabor, C.W. (1984). Expres-

sion of the Cloned Genes Encoding the Putrescine Biosynthetic Enzymes and Methionine Adenosyltransferase of *Escherichia coli* (*speA, speB, speC* and *metK*). *Gene*, *30*(1–3), 129–136. https://doi.org/10.1016/0378-1119(84)90113-6

Cui, S., Ling, P., Zhu, H., & Keener, H.M. (2018). Plant Pest Detection Using an Artificial Nose System: A Review. *Sensors (Switzerland)*, *18*(2), 1−18. https://doi.org/10.3390/s18020378

Debode, F., Marien, A., Janssen, E., Bragard, C., & Berben, G. (2017). The Influence of Amplicon Length on Real-time PCR Results. *Biotechnology, Agronomy, Society and Environment*, *21*(1), 3−11.

Edy, N., Subandiyah, S., Sumardiyono, C., & Widada, J. (2011). Karakterisasi dan Deteksi Cepat Bakteri Penyebab Penyakit Darah pada Pisang. *Jurnal Perlindungan Tanaman Indonesia, 17*(1), 26−30.

Erva, R.R., Varghese, R.P., Prasanna, A.L, Madhunika, G., Ravi Teja, K.S., Santosh, V.S., & Rajulapati, S.B. (2018). An In Silico Structural Study on Bacterial Sulfite Reductase. *Current Trends in Biotechnology and Pharmacy, 12*(4), 342−348.

European and Mediterranean Plant Protection Organization (EPPO). (2018). Diagnostics: PM7/21 (2) *Ralstonia solanacearum*, *R. pseudosolanacearum* and *R. syzygii* (Ralstonia solanacearum species complex). *Bulletin OEPP/EPPO Bulletin*, *48*(1), 32−63.

Fauque, G.D., & Barton, L.L. (2012). Hemoproteins in Dissimilatory Sulfate- and Sulfur- Reducing Prokaryotes. *Advances in Microbial Physiology* 60, 1−90.

Fegan, M. & Prior, P. (2005). How complex is the "*Ralstonia solanacearum* species complex"? In Allen, C., Prior, P., & Hayward, A.C. (Eds.), *Bacterial Wilt: The Disease and the* Ralstonia solanacearum *Species Complex* (pp. 449–461). St. Paul, Minnesota: American Phytopathological Society.

Gao, M., Tashiro, Y., Yoshida, T., Zheng, J., Wang, Q., Sakai, K., & Sonomoto, K. (2015). Metabolic Analysis of Butanol Production from Acetate in *Clostridium saccharoperbutylacetonicum* N1-4 Using 13C Tracer Experiments. *RSC Advances*, *5*(11), 8486−8495. https://doi.org/10.1039/C4RA09571E

Kalapos, M.P. (2014). Acetone. In Wexler, P. (Ed.), *Reference Module in Biomedical Sciences, from Encyclopedia of Toxicology* (3rd ed., pp. 36−39). London: Academic Press.

Kang, A., & Lee, T.S. (2015). Converting Sugars to Biofuels: Ethanol and Beyond. *Bioengineering*, *2*, 184−203. https://doi.org/10.3390/bioengineering2040184 OPEN

Kubota, R., Schell, M.A., Peckham, G.D., Rue, J., Alvarez, A.M., Allen, C., & Jenkins, D.M. (2011). In Silico Genomic Subtraction Guides Development of Highly Accurate, DNA-based Diagnostics for *Ralstonia solanacearum* Race 3 Biovar 2 and Blood Disease Bacterium. *Journal of General Plant Pathology*, *77*, 182–193. https://doi.org/10.1007/s10327-011-0305-2

Kushkevych, I., Cejnar, J., Treml, J., Dordević, D., Kollar, P., & Vítězová, M. (2020). Recent Advances in Metabolic Pathways of Sulfate Reduction in Intestinal Bacteria. *Cells*, *9*(698), 1−16. https://doi.org/10.3390/cells9030698

Kyoto Encyclopedia of Genes and Genomes (KEGG). (2021). Retrieved from https://www.genome.jp/entry/gn:T07219

Lenarčič, R., Morisset, D., Pirc, M., Llop, P., Ravnikar, M., & Dreo, T. (2014). Loop-mediated Isothermal Amplification of Specific Endoglucanase Gene Sequence for Detection of the Bacterial Wilt Pathogen *Ralstonia solanacearum*. *PLoS ONE*, *9*(4), e96027. https://doi.org/10.1371/journal.pone.0096027

Lowe-Power, T.M., Hendrich, C.G., von Roepenack-Lahaye, E., Li, B., Wu, D., Mitra, R., Dalsing, B.L., Ricca, P., Naidoo, J., Cook, D., Jancewicz, A., Masson, P., Thomma, B., Lahaye, T., Michael, A.J., & Allen, C. (2018). Metabolomics of Tomato Xylem Sap during Bacterial Wilt Reveals *Ralstonia solanacearum* Produces Abundant Putrescine, a Metabolite that Accelerates Wilt Disease. *Environmental Microbiology*, *20*(4), 1330–1349. https://doi.org/10.1111/1462-2920.14020

Prakoso, A.B., Anjani, A.A., Joko, T., Drenth, A., Soffan, A, & Subandiyah, S. (2020). Transcriptomic Data of the *Musa balbisiana* Cultivar Kepok Inoculated with *Ralstonia syzigii* subsp. *celebesensis* and *Ralstonia solanacearum*. *Data in Brief*, *29*, 105366. https://doi.org/10.1016/j.dib.2020.105366

Ray, J.D., Subandiyah, S., Rincon-Florez, V.A., Prakoso, A.B., Mudita, I.W., Carvalhais, L.C., Markus, J.E.R., O'Dwyer, C.A., & Drenth, A. (2021). Geographic Expansion of Banana Blood Disease in Southeast Asia. *Plant Disease*, *105*(10), 2792−2800. https://doi.org/10.1094/PDIS-01-21-0149-RE Epub 2021 Nov 9. PMID: 33973808.

Remenant, B., de Cambiaire, J.C., Cellier, G., Jacobs, J.M., Mangenot, S., Barbe, V., Lajus, A., Vallenet, D., Medigue, C., Fegan, M., Allen, C., & Prior, P. (2011). *Ralstonia syzygii*, the Blood Disease Bacterium and Some Asian *R. solanacearum* Strains form a Single Genomic Species Despite Divergent Lifestyles. *PLoS ONE*, *6*(9), 1−10. https://doi.org/10.1371/journal.pone.0024356

Rincón-Flórez, V.A., Ray, J.D., Carvalhais, L.C., O'Dwyer, C.A., Subandiyah, S., Zulperi, D., & Drenth, A. (2022). Diagnostics of Banana Blood Disease. *Plant Disease*, *106*(3), 947−959. https://doi.org/10.1094/PDIS-07-21-1436-RE. Epub 2022 Mar 10. PMID: 34668403.

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. s. *International Journal of Systematic and Evolutionary Microbiology*, *64*, 3087−3103. https://doi.org/10.1099/ijs.0.066712-0

Safni, I., Subandiyah, S., & Fegan, M. (2018). Ecology, Epidemiology and Disease Management of *Ralstonia syzygii* in Indonesia. *Frontiers in Microbiology*, *9* (MAR). https://doi.org/10.3389/fmicb.2018.00419

Schulz, S., & Dickschat, J.S. (2007). Bacterial Volatiles: The Smell of Small Organisms. *Natural Product Reports*, *24*(4), 814−842. https://doi.org/10.1039/b507392h

Spraker, J.E., Jewell, K., Roze, L.V., Scherf, J., Ndagano, D., Beaudry, R., Linz, J.E., Allen, C., & Keller, N.P. (2014). A Volatile Relationship: Profiling an Inter-Kingdom Dialogue Between Two Plant Pathogens, *Ralstonia Solanacearum* and *Aspergillus Flavus*. *Journal of Chemical Ecology, 40*(5), 502–513. https://doi.org/10.1007/s10886-014-0432-2

Talib, M.A.A., Azeme, N.F.N., Shafie, K.A., Sulastri, N., Jaafar, S.N.B., Haron, F.F., Mazlan, Z., Azlina, A., & Kadir, A. (2021). Lateral Flow Immunoassay Detection of *Ralstonia syzygii* subsp. *celebensis* Causing Banana Blood Disease in Symptomatic and Asymptomatic Banana Plants. *Journal of Tropical Plant Physiology, 13*(1), 51−62. Retrieved from https://jtpp.org.my/volume/13_1/vol13_1_article5.pdf

Teng, S.K., Aziz, N.A.A., Mustafa, M., Laboh, R., Ismail, I.S., Sulaiman, S.R., Azizan, A.A. & Devi, S. (2016). The Occurrence of Blood Disease of Banana in Selangor, Malaysia. *International Journal of Agriculture and Biology 18*(1), 92–97. https://doi.org/10.17957/IJAB/15.0067