Geographic Expansion of Banana Blood Disease in Southeast Asia

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Abstract

Blood disease in bananas caused by *Ralstonia syzygii* subsp. *celebesensis* is a bacterial wilt causing significant crop losses in Indonesia and Malaysia. Disease symptoms include wilting of the plant and red-brown vascular staining, internal rot, and discoloration of green banana fruit. There is no known varietal resistance to this disease in the *Musa* genus, although variation in susceptibility has been observed, with the popular Indonesian cooking banana variety Kepok being highly susceptible. This study established the current geographic distribution of Blood disease in Indonesia and confirmed the pathogenicity of isolates by Koch's postulates. The long-distance distribution of the disease followed an arbitrary pattern indicative of human-assisted movement of infected banana materials. In contrast, local or short-distance spread radiated from a single infection source, indicative of dispersal by insects and possibly contaminated tools, water, or soil. The rapid expansion of its geographical range makes Blood disease an emerging threat to banana production in Southeast Asia and beyond.

Keywords: disease development and spread, epidemiology, fruit, prokaryotes, tropical plants

Bananas are an important crop for developing countries in the tropics and subtropics, providing a staple food source and income for smallholder farmers, as well as an important global commodity and the world's most consumed fruit (Ploetz et al. 2015). Cultivated bananas are sterile hybrids among either *Musa balbisiana* Colla (B-genome) or *Musa acuminata* Colla (A-genome) subspecies or hybrids between the two (Simmonds and Shepherd 1955). Edible banana varieties are parthenocarpic, thus requiring clonal propagation through suckers (Robinson 1996).

Banana bacterial wilt diseases have a significant impact on banana production; they include Moko (*Ralstonia solanacearum* sensu stricto), Blood disease (*Ralstonia syzygii* subsp. *celebesensis*), and banana Xanthomonas wilt (*Xanthomonas vasicola* pv. *musacearum*) (Blomme et al. 2017). Prior to taxonomic reclassification by Safni et al. (2014), Blood disease was attributed to the Blood disease bacterium (BDB) in the *R. solanacearum* species complex (phylotype IV). Koch's postulates were not performed on the *R. syzygii* subsp. *celebesensis* reference isolates used in the 2014 taxonomic revision (Safni et al. 2014). The isolates from the taxonomic study by Safni et al. (2014) are a reference for the identity of *R. syzygii* subsp. *celebesensis,* and once the isolates are confirmed as pathogenic, then this taxon can be firmly established as the causal agent of Blood disease. Blood disease symptoms are characterized by leaves that wilt and become chlorotic or necrotic, red-brown internal vascular staining, and fruit bunches that appear outwardly healthy but internally the fruit pulp is discolored, rotten, and inedible (Blomme et al. 2017; Tjahjono and Eden-Green 1988).

Blood disease was first reported from the province of South Sulawesi, Indonesia, in the early 1900s (Gäumann 1921; Rijks 1916). Restricted movement of banana plant materials from Sulawesi prevented its dispersal for many years until it was detected in West Java in 1987 (Tjahjono and Eden-Green 1988). Blood disease was subsequently reported from several other islands in Indonesia, and it was detected in peninsular Malaysia (Safni et al. 2014; Supriadi 2005; Teng et al. 2016; Tjahjono and Eden-Green 1988). Indonesian disease reports are mostly from the larger islands; many are unconfirmed, based on symptoms only, with no identified bacterial agent, and represent only a few of the 17,000 islands of the Indonesian archipelago. As a result, the current geographic distribution of the disease is not well known.

Musa is the only known plant genus able to be infected naturally by *R. syzygii* subsp. *celebesensis*. In 1921, Gäumann reported that >100 different *Musa* varieties planted in a field site in Sulawesi in 1918 had succumbed to the disease by 1920. Among the varieties tested by Gäumann (1921), some variation in susceptibility was observed. Subsequent field observations have confirmed Indonesia's most important cooking banana, Kepok, to be highly susceptible (Buddenhagen 2009; Eden-Green 1994; Gäumann 1921). There is no current information on the *Musa* hosts grown across Indonesia that succumb to Blood disease.

Blood disease transmission at the local, or plant-to-plant, level is primarily attributed to insects mechanically transferring the bacteria from an infected to a healthy banana inflorescence (Buddenhagen 2009; Stover and Espinoza 1992). Local transmission is also hypothesized to occur through contaminated tools, soil, water, bats, and birds (Buddenhagen 2009; Gäumann 1923; Safni et al. 2018). In contrast, long-distance spread is associated with humans through the movement of infected banana plant materials (Gäumann 1921). Many aspects of the epidemiology and disease cycle of Blood disease remain uncertain because relatively little biological research has been conducted over the past 100 years.

Blood disease may pose a serious threat to banana production in Southeast Asia and beyond as it progresses from a pathogen of local economic importance on a few islands of the Indonesian archipelago to one that could become widely distributed throughout the Asian

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region and beyond. Therefore, the broad objectives of this study were to establish the past and present distribution and spread of Blood disease and to determine the identities of the pathogenic isolates associated with the disease. The specific questions we sought to address were these. 1) What is the current geographic distribution of Blood disease? 2) Are there any patterns in its short- and long-distance spread? 3) How are the symptoms expressed, and what banana hosts are commonly infected in the field? 4) What are the identities of the isolates associated with Blood disease? 5) Are the isolates from symptomatic plants able to satisfy Koch's postulates? Answers to these questions are critical to developing strategies to manage the disease in areas where it is endemic, and to prevent incursions into islands or regions currently free of the disease.

Materials and Methods

Surveys and sample collection. To confirm the past distribution, and to investigate the current distribution of *R. syzygii* subsp. *celebesensis*, a series of surveys were conducted in Indonesia from 2017 to 2019 (Table 1). The surveys were generally targeted at islands or areas with previous confirmed or unconfirmed reports of the disease.

Samples were collected from symptomatic *Musa* spp. plants growing on smallholder farms, on commercial farms, on private households and along roadsides. Disease symptoms were documented and the hosts were identified. For the island of Sumba, where an incursion had occurred, a more detailed survey was conducted, and farmers were asked when they had first noticed the symptoms in their plants. Samples of fruit, pseudostem, or petiole collected from symptomatic banana plants were placed into plastic bags and stored at room temperature. Bacterial isolations were usually carried out within 36 h of sample collection, with the exception of a few posted samples that were processed ≤10 days after collection. Only one isolate of *R. syzygii* subsp. *celebesensis* was selected from each banana mat for further analysis.

Bacterial isolation, maintenance, and storage of isolates. *Media used.* The bacteria were initially isolated, purified, and sustained on a modified tetrazolium chloride medium (TZC) in Petri dishes (Kelman 1954). Standard TZC medium is casamino acid peptone glucose medium (CPG; 10 g liter^{-1} of peptone, 1 g liter^{-1} of casein hydrolysate, 5 g liter^{-1} of D-glucose, 16 g liter^{-1} of bacto agar) with the addition of 50 mg liter^{-1} of 2,3,5 triphenyl tetrazolium chloride (TTC). The modified TZC used here contained a lower concentration of TTC to facilitate better growth of *R. syzygii* subsp. *celebesensis*. Two concentrations of TTC were assessed: 1/4 TZC (CPG + 12.5 mg liter^{-1} of TTC) and 1/2 TZC (CPG + 25 mg liter^{-1} of TTC), with 1/2 TZC being favored by the authors. Cyclohexamide (C; 100 mg liter^{-1}) was added to inhibit fungal growth during isolation.

Table 1. Targeted surveys and sampling sites for Blood disease (BD) to establish the occurrence of *Ralstonia syzygii* subsp. *celebesensis* across Indonesia

Island	Province	Month	Year	BD detected
Java	Central Java	October	2017	Yes
Java	Special Region of Yogyakarta	October	2017	Yes
Sulawesi	Southeast Sulawesi	October	2017	Yes
Sumba	East Nusa Tenggara	November	2017	Yes
Timor (West)	East Nusa Tenggara	November	2017	No
New Guinea	West Papua (Manokwari)	January	2018	Yes
New Guinea	Papua (Merauke)	October	2018	No
New Guinea	Papua (Jayapura)	October	2018	No
Bali	Bali	March	2018	Yes
Flores	East Nusa Tenggara	March	2018	No
Sumbawa	West Nusa Tenggara	April	2018	Yes
Borneo	East Kalimantan	September	2018	Yes
Borneo	North Kalimantan	September	2018	No
Borneo	West Kalimantan	September	2018	No
Sulawesi	North Sulawesi	March	2019	Yes
Sulawesi	South Sulawesi	March	2019	Yes
Kayuadi	South Sulawesi	March	2019	Yes
Selayar	South Sulawesi	March	2019	Yes
Tanah Jampea	South Sulawesi	March	2019	Yes
Borneo	South Kalimantan	September	2019	Yes
Borneo	Central Kalimantan	September	2019	Yes
Sumatra	Lampung	September	2019	Yes
Sumatra	North Sumatra	November	2019	Yes

For biochemical testing, DNA extraction, and inoculum production, the isolates of *R. syzygii* subsp. *celebesensis* were grown on CPG medium. The cultures were incubated at 28 ± 2°C (room temperature) in the dark.

Isolation of R. syzygii *subsp.* celebesensis *from banana tissues.* Samples were surface sterilized with 80% ethanol prior to isolation using aseptic techniques. From fruit, a 10-mm^2- × 4-mm-deep section with vascular staining was excised from the skin, cut into small pieces, placed into a 2-ml tube containing 1 ml of sterile water and incubated for 5 to 10 min. Using a 10-µl loop, approximately 60 µl of the inoculum was streaked onto a Petri dish containing 1/4- or 1/2-strength TZC + C to obtain single colonies. From pseudostems or petioles showing signs of infection in the form of lightly colored vascular staining, discolored vascular strands were excised from approximately 2 cm^2 of plant material, cut into small pieces, placed into a 2-ml tube containing 1 ml of sterile water, and incubated for 5 to 10 min. Then, approximately 40 µl of the inoculum was streaked onto medium as described above. A single bacterial colony matching the distinctive colony morphology and characteristics of *R. syzygii* subsp. *celebesensis* grown on 1/4- or 1/2-strength TZC was selected and streaked onto CPG medium in a Petri dish. Colonies were slow growing, round, red, with a white halo after 4 to 6 days of incubation. Where no single colony was available for selection, the isolate was restreaked onto 1/4 or 1/2 TZC + C medium until a single colony or pure culture could be obtained.

Reference isolates identified as *R. syzygii* subsp. *celebesensis* were retrieved from the Queensland Plant Pathology Herbarium. They were as follows: UQRS 479, UQRS 480, and UQRS 481. These isolates were included in this study as controls for the molecular identification because they had been used in the polyphasic taxonomic revision by Safni et al. (2014). They were grown on CPG medium. DNA was extracted as detailed below.

Storage of isolates. Isolates of *R. syzygii* subsp. *celebesensis* were grown in Petri dishes with CPG medium for 1 to 3 days. The cultures were stored in 2-ml microtubes containing 1 ml of sterile water in the dark at room temperature (Kelman and Person 1961), and in cryovials (Microbank, Prolab Diagnostics, Canada) at −80°C at the Research Center for Biotechnology UGM, Yogyakarta, Indonesia.

Biochemical testing. Isolates of *R. syzygii* subsp. *celebesensis* selected on the basis of colony morphology were initially screened using two biochemical tests. Bacterial isolates were grown on CPG medium for 1 to 3 days. They were characterized as gram negative (G−ve) or gram positive (G+ve) using the presumptive potassium hydroxide test (Schaad et al. 2001). The isolates were then classified as oxidase positive or oxidase negative. The ability to produce the cytochrome oxidase enzyme separates *Pseudomoadaceae* (oxidase positive) from *Enterobacteriaceae* (oxidase negative). A strong color change on oxidase detection strips (MB0266, Oxoid, Thermo Fisher Scientific) indicates the production of oxidase. *Ralstonia syzygii* subsp. *celebesensis* is gram negative and oxidase positive.

DNA extraction and molecular identification. DNA was extracted using the Isolate II genomic DNA Kit (Bioline) according to the manufacturer's instructions with the following alterations. To prevent blockage of the spin column and ensure liquid flow through following the adjustment of DNA binding conditions, an additional step was added. The sample was centrifuged for 1 min at 2,700 × *g*, and the supernatant was then transferred to the Isolate II Genomic DNA spin column. All centrifugation was carried out for 1.5 min at 15,300 × *g* at room temperature.

Specific R. syzygii *subsp.* celebesensis *PCR.* To confirm the identity of the isolates, a PCR specific for *R. syzygii* subsp. *celebesensis* was conducted for all Indonesian isolates and the reference isolates UQRS 479, UQRS 480, and UQRS 481 using previously designed primers (Das 2004; Tan 2003). The primer sequences were originally reported with the directions reversed and are listed here with the correct direction 121F (5′-AAGTTCATTGGTGCCGAATCA-3′) and 121R

(5′-CGTATTGGATGCCGTA**A**TGGA-3′). An alignment between the reverse primer and the originally reported sequence data showed a difference in the nucleotide, resulting in the corrected primer sequence 121R (5′-CGTATTGGATGCCGTA**T**TGGA-3′). In this study we used both reverse primers listed here with equivalent results. Each PCR reaction contained 0.4 µM each primer, 1 U of MyTaq HS DNA polymerase (Bioline), 5× MyTaq HS Red Reaction buffer (containing 1 mM dNTPs and 3 mM $MgCl_2$), and 1 ng of DNA template in a total of 20 µl. The cycling conditions consisted of an initial denaturation of 95°C for 1 min, followed by 30 cycles of denaturation of 95°C for 15 s, annealing at 57°C for 15 s, and elongation at 72°C for 10 s. PCR products were visualized by electrophoresis on a 1.5% agarose gel. The presence of a 317-bp amplicon would indicate the presence of *R. syzygii* subsp. *celebesensis.* Reactions included both negative (water template) and a positive control.

Endoglucanase gene sequencing. To confirm the identities of the isolates based on the biochemical tests and the specific *R. syzygii* subsp. *celebesensis* PCR, a region of the endoglucanase (*egl*) gene was amplified and sequenced according to previously published methodology for a representative isolate from each location (Fegan et al. 1998). Sequencing and analysis of the *egl* gene was used to confirm the identities of the isolates as *R. syzygii*. Specifically, primers Endo-F (5′-ATGCATGCCGCTGGTCGCCGC-3′) and Endo-R (5′-GCG TTGCCCGGCACGAACACC-3′) were used to amplify a 750-bp amplicon from the *egl* gene region. The PCR reaction contained 0.4 µM each primer, 1 U of MyTaq HS DNA polymerase (Bioline), 5× MyTaq HS Red Reaction buffer, and 1 ng of DNA template in a final volume of 20 µl per reaction. The cycling conditions consisted of an initial denaturation step at 95°C for 1 min, followed by 30 cycles of denaturation at 95°C for 15 s, annealing at 70°C for 15 s, and elongation at 72°C for 10 s. The amplified PCR products were visualized using gel electrophoresis, and PCR products were sequenced using the direct Sanger method (Macrogen, South Korea). The raw sequence data were analyzed using Geneious v. 10.2.6 and aligned with available sequences in GenBank using BLAST (Zhang et al. 2000). Sequence data from isolates representative of each location were deposited into the GenBank database (Table 2). DNA was imported to Australia under Australian Government Department of Agriculture and Water Resources import permit numbers 0001092808 and 0002946377.

Temporal distribution of Blood disease in Indonesia. To determine the dissemination of Blood disease over time, a detailed analysis of culture collections and reports written in Dutch, Indonesian, and English over the past century was conducted to determine past and present distributions for *R. syzygii* subsp. *celebesensis* and patterns of distribution in Indonesia. A survey conducted by Gäumann (1921) documented the distribution of Blood disease in South Sulawesi, and the report contains a map (see figure 2 in Gäumann 1923). Data from this map are included in our analysis. Because these earlier records are not supported by isolates held in culture collections, extensive surveys were conducted in southern Sulawesi and the Selayer Islands to determine the presence of the disease in these areas and to confirm that the historical records were likely to represent Blood disease.

Koch's postulates. To confirm that the isolates verified as *R. syzygii* subsp. *celebesensis* using molecular diagnostics were the causal organism of Blood disease, Koch's postulates were conducted in Indonesia using five isolates (JR3412, JR3461, JR3759, JR3824, and JR3825) of *R. syzygii* subsp. *celebesensis* obtained from diverse geographic origins.

Table 2. Validated geographic locations of Blood disease from isolates collected during this study and from Queensland Plant Pathology Herbarium (BRIP) confirmed as *Ralstonia syzygii* subsp. *celebesensis*[a]

Island	Province	Regency	Identifier	GenBank accession
Bali	Bali	Klunkung	JR3532B1	MT984305
Bali	Bali	Gianyar	JR3539A2	MT984306
Bali	Bali	Tabanan	BRIP60800a/UQRS480	KC757105
Borneo	Central Kalimantan	Seruyan	JR3798	MT984307
Borneo	East Kalimantan	Balikapapan	JR3589	MT984308
Borneo	East Kalimantan	Kutai Kartanegara	JR3600	MT984309
Borneo	South Kalimantan	Banjar	JR3814	MT984310
Borneo	South Kalimantan	Kota Banjarbaru	JR3800	MT984311
Borneo	South Kalimantan	Tanah Laut	JR3805	MT984312
Borneo	South Kalimantan	Tapin	JR3820	MT984313
Java	Central Java	Magelang	JR3416	MT984314
Java	West Java	–	BRIP60839a	KC757112
Java	Yogyakarta[b]	Sleman	JR3412	MT984315
Java	Yogyakarta[b]	Kulon Progo	JR3420	MT984316
Java	Yogyakarta[b]	Bantul	JR3824	MT984317
Kayuadi	South Sulawesi	Selayar	JR3759	MT984318
New Guinea	West Papua	Manokwari	JR3825	MT984319
Selayar	South Sulawesi	Selayar	JR3729	MT984320
Sulawesi	South Sulawesi	Kota Makassar	JR3696	MT984321
Sulawesi	South Sulawesi	Gowa	JR3717A	MT984322
Sulawesi	South Sulawesi	Maros	JR3703	MT984323
Sulawesi	South Sulawesi	Takalar	JR3714	MT984324
Sulawesi	Southeast Sulawesi	Kota Kendari	JR3827	MT984325
Sulawesi	North Sulawesi	Kota Manado	JR3671	MT984326
Sulawesi	North Sulawesi	Minihasa Utara	JR3694	MT984327
Sulawesi	North Sulawesi	Minihasa	JR3675	MT984328
Sulawesi	North Sulawesi	Kota Tomohon	JR3684	MT984329
Sumatra	North Sumatra	Deli Serdang	JR3828	MT984330
Sumatra	North Sumatra	Kota Medan	JR3830	MT984331
Sumatra	South Sumatra	Lampung	JR3833	MT984332
Sumba	East Nusa Tenggara	East Sumba	JR3483	MT984333
Sumba	East Nusa Tenggara	Southwest Sumba	JR3461	MT984334
Sumba	East Nusa Tenggara	West Sumba	JR3466	MT984335
Sumbawa	West Nusa Tenggara	Sumbawa	JR3831	MT984336
Tanah Jampea	South Sulawesi	Kepulauan Selayar	JR3756	MT984337

[a] The identity of representative isolates are confirmed using biochemical and molecular diagnostics. Isolates were classed as gram negative and oxidase positive using a presumptive gram potassium hydroxide test and oxidase detection strips (MB0266, Oxoid) (Schaad et al. 2001). Isolates identified with specific primers (121F/121R), and partial endoglucanase gene (*egl*) sequence analysis (Das 2004; Fegan et al. 1998). Partial endoglucanase gene (*egl*) sequences were deposited in GenBank.

[b] Special Region of Yogyakarta.

Banana test plants. Commercially produced Kepok "Kuning" plants originating from tissue culture and grown in potting mix in 30-cm-diameter pots (Institute Plants Centre, Magelang, Central Java, Indonesia) were used to test Koch's postulates. Plants were raised in a shade house at the Research Center for Biotechnology UGM, Yogyakarta, in natural light with a maximum day temperature of 38°C and a minimum night temperature of 23°C, and watered regularly prior to inoculation at the nine-leaf growth stage.

Inoculum. Bacterial isolates used in this experiment were retrieved from cultures stored in microtubes containing sterile water. Isolates were revived by streaking onto 1/2 TZC medium in Petri plates; after 4 days, single colonies were selected and cultured on CPG medium in Petri plates for 2 days. Cultures were grown at 28 ± 2°C (room temperature) in the dark. Inoculum was prepared by suspending 2-day-old colonies in sterile deionized water and adjusting to approximately 10^8 CFU ml^{-1} using a spectrometer (OD600 Implen, Munich, Germany). Following inoculation, the inoculum was serially diluted to confirm viability and concentration.

Inoculation. Plants were inoculated by injecting 500 µl of the inoculum using a 25-gauge needle into the pseudostem approximately 3 cm above the soil line. The treatments comprised five isolates, JR3412, JR3461, JR3759, JR3824, and JR3825, and a water control. Each treatment included three plants.

Disease assessment, isolation, and confirmatory PCR. Plants were assessed for external symptoms and harvested 17 days after inoculation for assessment of internal symptoms. Plants showing symptoms of leaf yellowing, necrosis, wilt, and internal vascular discoloration were considered symptomatic. Isolations were carried out as described previously, from selected discolored vascular strands at the top of the pseudostem at least 10 cm away from the inoculation point. Bacteria with characteristic colony morphology were identified using the specific *R. syzygii* subsp. *celebesensis* PCR test based on the 121-primer pair as described above.

Results

Current distribution. Our surveys revealed the presence of *R. syzygii* subsp. *celebesensis* in many locations across Indonesia, including the province of South Sulawesi and all of the Selayar Islands surveyed (Table 1). For several locations, the historical records were confirmed or the first record was established, supported by identified cultures (Tables 2 and 3). The surveys confirmed, for the first time, the presence of Blood disease in the province of Special Region of Yogyakarta, Southeast Sulawesi, West Papua, Central Kalimantan, and South Kalimantan and on the island of Sumba in the province of East Nusa Tenggara (Table 3).

It was not detected on the island of Flores, West Timor, the cities of Merauke and Jayapura in the Papua province, or the provinces of West Kalimantan and North Kalimantan (Table 1).

Dissemination pattern. The literature review and data from this survey provided a detailed picture of the pattern of distribution of Blood disease over time (Fig. 1; Table 3). When plotted on a map, the long-distance dispersal did not radiate from a particular source but followed an arbitrary pattern (data not shown). A more detailed analysis of the incursion of Blood disease on the island of Sumba revealed that local distribution from a single infection site spread rapidly into areas previously free of the disease. The disease epidemic on Sumba radiated from a single infection site to contiguous banana plantings over time. Within 4 years, the disease was widespread, and local farmers were reporting losses of 75 to 100% (Fig. 2).

Symptoms and host identity. A characteristic symptom of Blood disease is the desiccation or rot of the male bell of the banana inflorescence (Fig. 3B and C). Vascular staining progresses from the diseased male bell through the peduncle toward the fruit. Once the disease reaches the fruit, the inside of the fruit discolors, becoming red, brown, or yellow, and rots, while the outside remains green and asymptomatic (Fig. 3D). Vascular staining progresses through the peduncle into the true stem (Fig. 3F and G). After reaching the corm, vascular staining can be seen in the pseudostem leaf sheaths and tends to be concentrated toward the center of the pseudostem (Fig. 3G). As the disease progresses the leaves turn brown and wilt, and fruit appears to ripen unevenly (Fig. 3A).

A different pattern of disease progression was sometimes observed wherein plants showed leaf chlorosis and wilting as the first external symptoms. This could be observed at any stage of plant growth but was more commonly observed in young plants prior to fruiting. Sometimes plants wilted and died before reaching maturity. In some of these cases, the symptomatic plant was found to be growing from the mat of a diseased mother plant. Sometimes in varieties other than Kepok, all shoots arising from the same mat wilted and died.

Symptoms of Blood disease were observed and confirmed as *R. syzygii* subsp. *celebesensis* from 18 varieties of *Musa* spp. and were most often found on the most commonly grown banana variety, Kepok (Table 4). *R. syzygii* subsp. *celebesensis* was identified on varieties within *Musa* × *paradisiaca* Linn., *Musa acuminata*, *Musa balbisiana*, and *Musa textilis.* The disease was confirmed from Kepok "Tanjung," which lacks a male bell; from Kepok "Sayang," which generally lacks a male bell but produces the occasional bunch with a bell; and from the variety Raja, which has retained bracts (Table 4).

Identities and pathogenicity of isolates. The identities of the isolates from diseased plants were confirmed as *R. syzygii* subsp. *celebesensis* by a combination of methods that included cultural morphology, biochemical tests, and molecular diagnostics. All isolates were gram negative and oxidase positive and showed characteristic colony morphology when grown on modified TZC medium. Colonies were slow growing, nonfluidal, circular, raised, and red, with an entire white margin (Fig. 4). Sequencing and analysis of the partial endoglucanase

Table 3. The first detection of blood disease in a geographic area represented by published records or cultures of *Ralstonia syzygii* subsp. *celebesensis* held in collections[a]

Island	Province	Date first observed	Reference
Selayar islands	South Sulawesi[b]	1905	Rijks 1916
Sulawesi	West Sulawesi	1907	Gäumann 1921
Sulawesi	North Sulawesi[b]	1987	URI collection[c]
Java	West Java[b]	1987	Eden-Green and Sastraatmadja 1990; Tjahjono and Eden-Green 1988
Java	Central Java[b]	1988	Supriadi 2005
Sumatra	South Sumatra[b]	1991	Kusumoto et al. 2004
Sumatra	Lampung	1993	Supriadi 2005, URI collection[c]
Borneo	East Kalimantan[b]	1994	Baharuddin 1994
Maluku Islands	Maluku	1994	Baharuddin 1994
Bali	Bali[b]	1994	Supriadi 2005
Java	East Java	1997	Supriadi 2005
Lombok	West Nusa Tenggara	1998	Supriadi 2005
Sumbawa	West Nusa Tenggara[b]	1998	Supriadi 2005
New Guinea	Papua (Timika)	1999	Davis et al. 2000
Ambon	Maluku	2005	Supriadi 2005
Sumatra	North Sumatra[b]	2005	Supriadi 2005
Sumatra	West Sumatra	2005	Supriadi 2005, URI collection[c]
Sumatra	Aceh	2007	Buddenhagen 2009
Peninsular Malaysia	Selangor	2013	Teng et al. 2016
Java	Special Region of Yogyakarta	2017	This study
Sulawesi	Southeast Sulawesi	2017	This study
Sumba	East Nusa Tenggara	2012	This study
New Guinea	West Papua (Manokwari)	2018	This study
Borneo	Central Kalimantan	2019	This study
Borneo	South Kalimantan	2019	This study

[a] Many of the early reports that were unvalidated because they lacked confirmatory molecular diagnostics and/or a stored culture were confirmed during this current study.
[b] Locations previously reported and confirmed during this study.
[c] Culture stored at University of Reunion Island culture collection (URI collection).

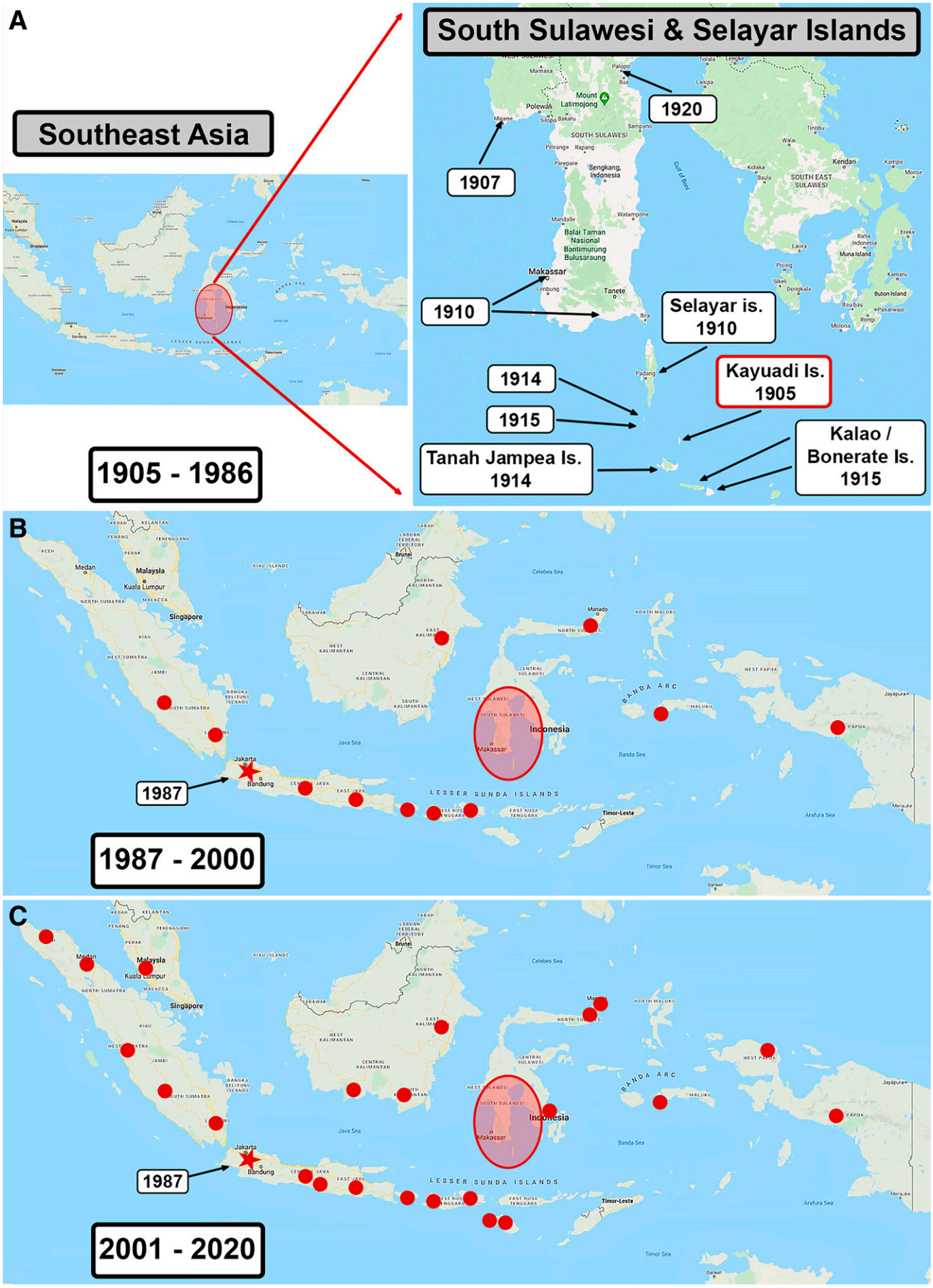

Fig. 1. Pattern of distribution over time for banana Blood disease caused by *Ralstonia syzygii* subsp. *celebesensis* (red circles/star/oval). **A,** First documented from Kayuadi Island, Indonesia, in 1905 the disease was contained by quarantine to Sulawesi for approximately 60 years. **B,** First recorded in West Java in 1987 (star) and spread to many locations by 2000. **C,** Current known distribution in 2020. Background, Google Maps (2020).

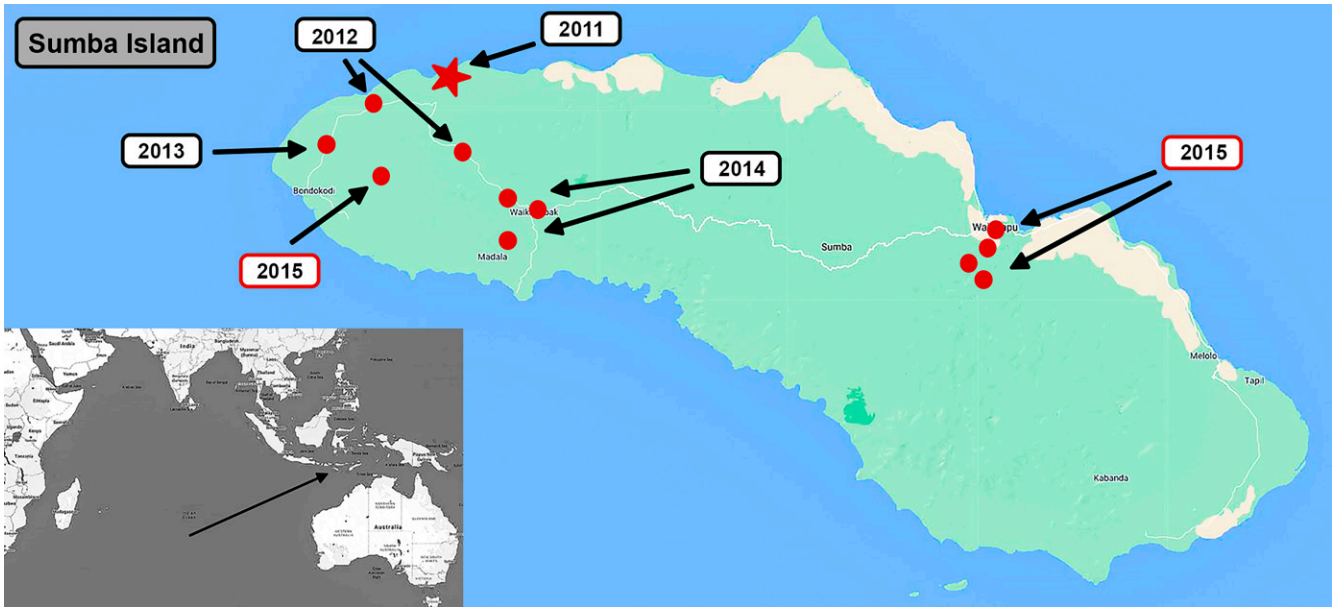

Fig. 2. Pattern of distribution over time on the island of Sumba, Indonesia for banana Blood disease caused by *Ralstonia syzygii* subsp. *celebesensis*. The disease was first noticed in 2011 (red star), confirmed locations and date of first occurrence (red circles) are depicted. Inset, Arrow denotes location of Sumba island. Background, Google Maps (2020).

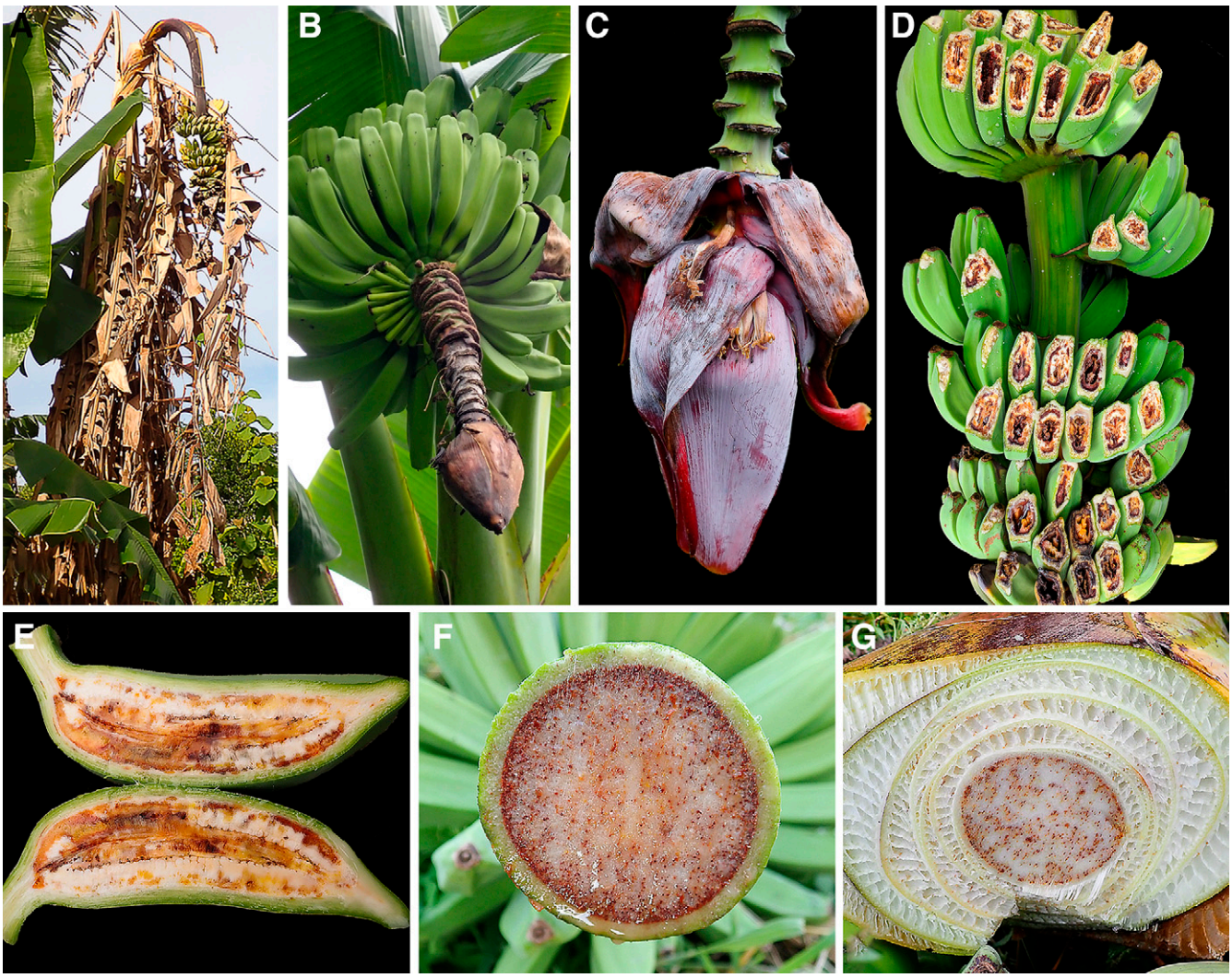

Fig. 3. Characteristic symptoms of Blood disease in Kepok banana plants caused by *Ralstonia syzygii* subsp. *celebesensis*. **A,** Banana plant with advanced symptoms showing leaf necrosis and wilt. **B and C,** Male banana bell with early symptoms of retained bracts and lower rachis and bell necrosis. **D and E,** Green fruit with discolored pulp. **F**, Bunch peduncle with vascular staining. **G,** Vascular staining in pseudostem.

gene region confirmed that the isolates were within the *R. syzygii* complex, and the Blood disease-specific PCR test described above verified the isolate identity as *R. syzygii* subsp. *celebesensis* (Table 2).

The isolates were confirmed as the causal agent of Blood disease by satisfying Koch's postulates. Inoculation of young potted plants with five isolates of *R. syzygii* subsp. *celebesensis* resulted in characteristic symptoms of wilt and vascular staining in all banana plants after 17 days of incubation, whereas the control plants remained asymptomatic (Fig. 5). The identity of the reisolated bacteria was confirmed using the Blood disease specific molecular test as described above.

Discussion

Survey results, combined with molecular analysis of the obtained isolates, revealed that Blood disease is common on several of the large islands of the Indonesian Archipelago, with only a few of the larger islands still apparently free of the disease. Blood disease was commonly found in South Sulawesi and on all the islands of the Selayar Island group surveyed, those being the localities where the disease was first reported over a century ago to cause significant damage. Many banana varieties surveyed were affected by the disease, including some that were previously deemed resistant because of the absence of a male bell. Molecular analysis confirmed the identities of the isolates from those plants as *R. syzygii* subsp. *celebesensis*, and reinoculations of healthy plants (Koch's postulates) confirmed their pathogenicity.

The first reports of Blood disease were from a commercial Kepok (Bluggoe subgroup) banana plantation on Kayuadi Island in the province of South Sulawesi in 1905 (Rijks 1916). Subsequently the disease was found on Selayar island in 1910, and it was later detected on the neighboring islands of Tanah Jampea, Kalao, and Bonerate in 1914 and 1915 (Rijks 1916). Bananas had been successfully grown in plantations on those islands and the fruit was shipped to markets in Makassar on the mainland for over 30 years prior to the Blood disease outbreak (Rijks 1916). The disease caused an epidemic that destroyed the industry on these islands within a few years (Rijks 1916). The nature of the epidemic that occurred in the Selayar Islands suggests that it was a disease incursion likely caused by movement of diseased banana planting material from elsewhere.

Gäumann (1921, 1922, 1923) conducted the first detailed investigations into Blood disease, looking into its distribution, biology, and control. Gäumann (1921), based at Madjene in West Sulawesi, reported that Blood disease was already widespread on cultivated banana plants in the area from 1907. He reported that the local villagers were very familiar with the disease and would routinely dig out diseased banana plants from their farms and leave them on the roadside to dry out in the sun as a control measure (Gäumann 1921). More wide-ranging surveys by Gäumann showed that the disease was widespread in South Sulawesi, West Sulawesi, and Southeast Sulawesi and on wild banana plants growing in virgin rainforest at altitudes below 1,200 to 1,300 m (Gäumann 1923). There are also historical anecdotal reports of sporadic outbreaks of a banana disease around Manado in North Sulawesi (Van Hall 1919), but these were not considered to be Blood disease (Gäumann, 1923). The current surveys revealed that Blood disease was common in all areas visited in Sulawesi (Table 1). All available data support the hypothesis of Gäumann (1921) that Blood disease is endemic to mainland southern Sulawesi, its likely center of origin, and that the disease was brought to the Selayar Islands in 1905 (Rijks 1916) several decades after banana plantations were established there.

Based on the recommendations of Gäumann (1921) for the management of Blood disease, the Dutch colonial administration imposed a quarantine in the 1920s that restricted the movement of banana fruits and planting materials out of Sulawesi. This restriction contained the pathogen for >60 years until the outbreak in West Java in 1987 (Buddenhagen 2009; Gäumann 1921; Tjahjono and Eden-Green 1988).

Table 4. Farm- or yard-grown banana varieties (*Musa* spp.) identified during this study with symptoms of Blood disease (BD) confirmed to be caused by *Ralstonia syzygii* subsp. *celebesensis*

Species	Variety/common name	Genotype	Number confirmed BD
Musa × *paradisiaca* Linn.	Kepok[a]	ABB	110
Musa × *paradisiaca* Linn.	Kepok "Tanjung"[b]	ABB	9
Musa × *paradisiaca* Linn.	Kepok "Sayang"[c]	ABB	2
Musa × *paradisiaca* Linn.	Raja Nanka	ABB	1
Musa × *paradisiaca* Linn.	Awak	ABB	5
Musa × *paradisiaca* Linn.	Raja	AAB	4
Musa × *paradisiaca* Linn.	Goroho	AAB	1
Musa acuminata Colla	Ambon	AAA	4
Musa acuminata Colla	Barangan	AAA	1
Musa acuminata Colla	Susu	AAA	5
Musa acuminata Colla	Tembaga Hijau	AAA	1
Musa acuminata Colla	Cavendish	AAA	5
Musa acuminata Colla	Gros Michel	AAA	1
Musa acuminata Colla	Acuminata var. Tomentosa	AA	1
Musa acuminata Colla	Mas	AA	2
Musa balbisiana Colla	Batu	BB	1
Musa textilis Née	Abacá		1
Musa spp.	Unknown		1

[a] Variation exists within Kepok, only Kepok "Tanjung" and Kepok "Sayang" have been discriminated.
[b] Lacking male bell.
[c] Limited or reduced stem numbers with male bell formed.

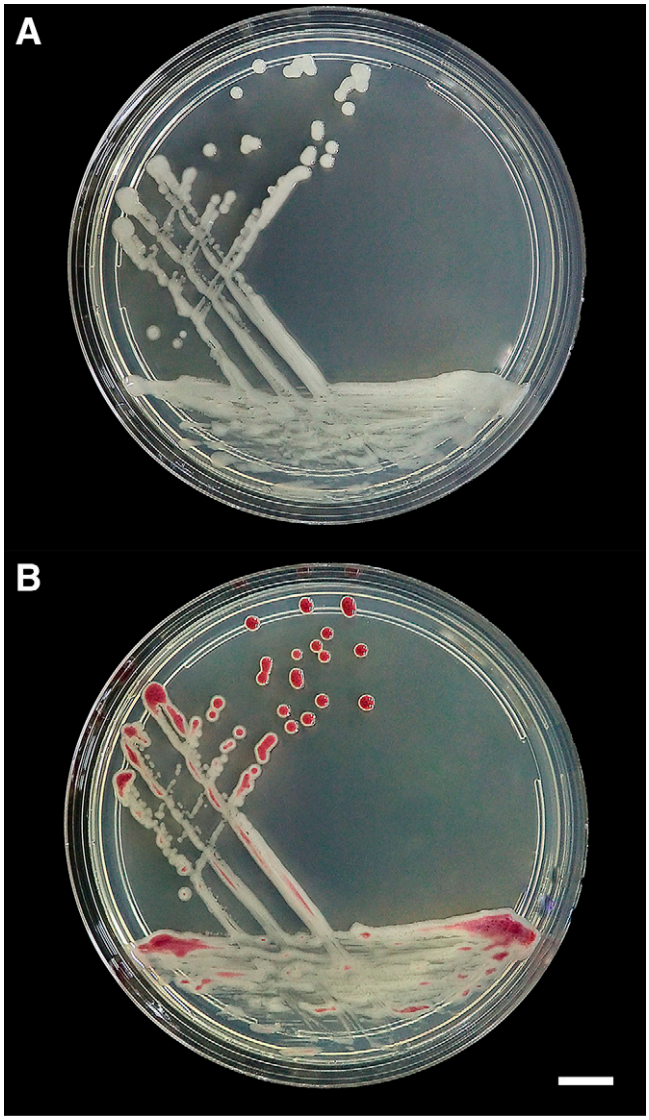

Fig. 4. Culture morphology of *Ralstonia syzygii* subsp. *celebesensis* isolate JR3824 grown on agar media in Petri dish after 5 days at 28°C. **A,** Growth on casamino acid peptone glucose medium (CPG). **B,** Growth on CPG medium amended with tetrazolium chloride and cyclohexamide 1/2 TZC + C. Scale bar = 10 mm.

Once the pathogen was present in Java, the movement of people from this highly populated island to less populated regions such as Papua, Kalimantan, and Sumatra may have inadvertently assisted its spread (Ploetz et al. 2015) and given rise to the arbitrary distribution pattern over the past three decades. Movement of banana suckers or plant materials is the most likely mode of long-distance dispersal for Blood disease, as is the case with Moko (Blomme et al. 2017; Gäumann 1921; Sequeira 1958). Another possible pathway of dispersal for bacterial wilts in banana, and one that requires further investigation, concerns infected fruit that may be discarded after transport, where they attract insects acting as vectors (Nakato et al. 2013).

Once the pathogen has become established in a new area, the most likely method of local movement is by insects mechanically transmitting the organism to nearby banana plants, causing an expanding disease front with potentially devastating results. For example, on Sumba Island, the disease was first observed in 2011 on the west side of the island. Over time the disease radiated from this initial infection point to eventually colonize most of the island. Within 4 years the profitable production and shipping of Kepok bananas had ceased. This pattern of long-distance spread followed by secondary spread from a newly established site follows a pattern known as the bridgehead effect (Lombaert et al. 2010) and has been shown to apply to many banana diseases (Drenth and Kema 2021).

The local spread of Blood disease radiating from a single source of infection suggests dispersal consistent with the disease being vectored by insects capable of flying short distances between host plants, and/or spread by contaminated tools, soil, or water within smallholders' plots. The distribution data suggest that the disease spreads locally only in the presence of the host plant. Disease spread is limited by areas of nonhost plants such as undeveloped natural forest and oil palm plantations, or by geography such as the boundaries of an island. The data suggested that long-distance spread in the absence of contiguous banana plantations is sporadic in nature and requires human assistance via the movement of infected planting materials or fruit.

During the current study, early symptoms of Blood disease were commonly observed in Kepok bananas in the form of the male bell becoming desiccated, and internal vascular staining that progressed from the male bell. These observations support the hypothesis that infection frequently starts through the male bell (Hadiwiyono 2011; Stover and Espinoza 1992). In other studies, droplets of bacteria were observed to ooze from the diseased male bell, which is routinely visited by insects (Buddenhagen 2009; Hadiwiyono 2011; Leiwakabessy 1999; Sahetapy 2013). The observation that the male bell is affected prior to wilting of the leaves supports the hypothesis that the male bell plays a major role in disease epidemiology, enabling rapid local dissemination of the causal agent from diseased to healthy bells by visiting insects (Buddenhagen 2009).

It has been reported that banana varieties such as Raja that retain bracts, or varieties such as Kepok "Tanjung" that do not produce a male bell, do not become diseased in the field and that these morphological features enable escape from infection (Buddenhagen 2009; Hermanto and Emilda 2013). However, the current study confirmed infection of both Kepok "Tanjung" and Raja. In these instances, infection may have occurred through insects visiting the female flowers or bract scars, or through contaminated tools, soil, or water, although conclusive evidence of these infection pathways is lacking. Detailed experimentation is required to elucidate the infection biology and epidemiology of Blood disease.

The extent of pathogenicity of *R. syzygii* subsp. *celebesensis* on different *Musa* species and subspecies has not been determined. Observations by Gäumann (1921) revealed that >100 different banana varieties, planted in field sites in Makassar and Watampone in 1918, had succumbed to the disease by 1920. Our field survey confirmed Blood disease from 18 different varieties of banana, including abaca (*M. textilis*), which is indicative of high levels of susceptibility across *M. textilis*, *M. acuminata*, and *M. balbisiana* species and subspecies and their hybrids. Thus far, no true resistance has been reported, although some *Musa* species are more susceptible to infection than others. Field trials are needed to identify whether any resistance or incomplete systemicity (bacteria does not colonize corm or suckers) exists within *Musa* spp. Such a finding may lead to improved disease management options.

Bell-less banana varieties such as Kepok "Tanjung," which originated from the Moluccas, and Kepok "Puju," which originated from Makassar, have been proposed as a solution to Blood disease (Buddenhagen 2009; Hermanto and Emilda 2013). This study demonstrated that Kepok "Tanjung" can become infected with Blood disease in the field. Kepok "Puju" is reported to occasionally produce stems with male bells, hence is also likely to be susceptible to infection by Blood disease. The Kepok banana has a sweet flavor, whereas the varieties without bells are sweet and a little sour, which is accepted by some consumers in certain regions but not accepted as a replacement for the popular Kepok in many areas of Indonesia. The Kepok banana remains popular and is commonly produced in tissue culture and grown commercially by smallholders across Indonesia, indicating that field management options for Blood disease are urgently required.

Our study found that certain areas and whole islands in the Indonesian archipelago are still free of the disease, and efforts to control or contain the disease are urgently required to reduce its impact and limit further spread. The effectiveness of quarantine, restricting the movement of field-grown planting material, is evidenced by the initial quarantine order from the 1920s, which saw Blood disease confined to parts of Sulawesi for >60 years. Therefore, efforts to reduce the movement of planting material and fruits from disease-affected areas may aid in

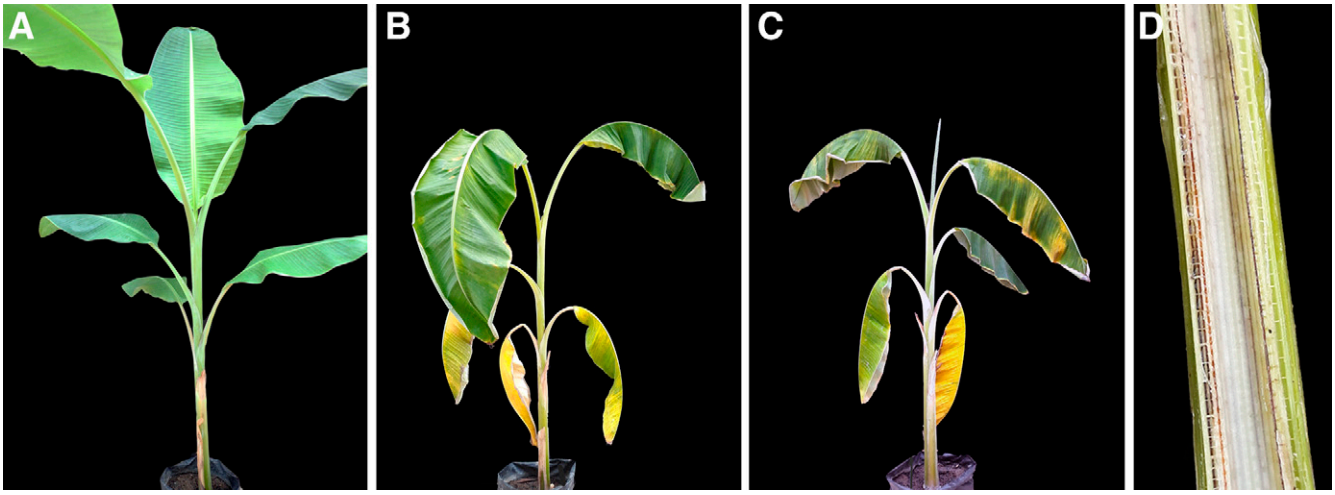

Fig. 5. Koch's postulates completed for *Ralstonia syzygii* subsp. *celebesensis* by stem injection of inoculum into potted Kepok banana plants to confirm causality of Blood disease. Plants assessed for symptoms of wilt and vascular staining after 17 days. **A**, Control mock inoculated. **B**, Wilted plant inoculated with JR3759 ex Kayuadi. **C**, Wilted plant inoculated with JR3461 ex Sumba. **D,** Vascular staining in pseudostem of plant inoculated with JR3461.

reducing the spread of the disease. Local management practices such as male bell removal using a special tool such as a forked stick, sanitation of tools, and removal with subsequent destruction of infected plants would likely reduce local spread of the disease (Blomme et al. 2009; Buddenhagen and Elsasser 1962; Hermanto and Emilda 2013).

Blood disease was first described more than a century ago and has significantly expanded its geographic range over the past 30 years, but little progress has been made in understanding its epidemiology or disease cycle. By completing Koch's postulates combined with molecular identifications validated using the reference isolates from the taxonomic revision by Safni et al. (2014), the current study has provided firm evidence that Blood disease is caused by *R. syzygii* subsp. *celebesensis.* Some of the knowledge gaps about Blood disease have been highlighted, and further research is under way to clarify the role of contaminated soil, water, and tools and to elucidate the role of the male and female inflorescences in the disease cycle of *R. syzygii* subsp. *celebesensis*. Greater clarity of the role of these components in local transmission of the disease may assist in the development of effective management approaches to control Blood disease of banana and ultimately reduce the impact of the disease.

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