Transmission of Blood disease in banana

Jane D. Ray[1], Siti Subandiyah[2,3], Ady B. Prakoso[3], Vivian A. Rincón-Flórez[1], Lilia C. Carvalhais[1], André Drenth[1]

[1]Centre for Horticultural Science, The University of Queensland, Brisbane, 4001, Australia,
[2]Research Center for Biotechnology, Universitas Gadjah Mada, Yogyakarta, 55281, Indonesia.
[3]Department of Entomology and Plant Pathology, Universitas Gadjah Mada, Yogyakarta, 55281, Indonesia.

Abstract

Banana Blood disease is a bacterial wilt caused by *Ralstonia syzygii* subsp. *celebesensis* and is an economically important disease in Indonesia and Malaysia. Transmission of this pathogen is hypothesized to occur through insects mechanically transferring bacteria from diseased to healthy banana inflorescences, and other pathways involving pruning tools, water movement and root-to-root contact. This study demonstrates that the ooze from the infected male bell and the sap from various symptomatic plant parts are infective and the cut surfaces of a bunch peduncle, petiole, corm, and the rachis act as infection courts for *R. syzygii* subsp. *celebesensis.* In addition, evidence is provided that *R. syzygii* subsp. *celebesensis* is highly tool transmissible, that the bacterium can be transferred from the roots of a diseased plant to the roots of a healthy plant and transferred from the mother plant to the sucker. We provide evidence that local dispersal of Blood disease is predominantly through mechanical transmission by insects, birds, bats or human activities from diseased to healthy banana plants and that long-distance dispersal is through the movement of contaminated planting material. Disease management strategies to prevent crop losses associated with this emerging disease are discussed based on our findings.

Keywords

Prokaryotes, tropical plants, disease development and spread, fruit, disease management.

Introduction

Bananas (*Musa* spp.) are a significant global commodity. The largest producing countries are India, China, and Indonesia, where bananas are traded and an important domestic food source (FAOSTAT 2018). Cultivated bananas are sterile intra- or inter-specific hybrids among and between *Musa acuminata* Colla (A-genome) and/or *Musa balbisiana* Colla (B-genome) (Simmonds and Shepherd 1955). The banana variety Cavendish (AAA) dominates the world markets, but in Indonesia, the cooking banana Kepok (ABB) is the main banana variety grown and traded by smallholder farmers (Buddenhagen 2009; Ray et al. 2021b).

The banana plant is a large perennial herbaceous monocotyledon with leaves that overlap in basal sheaths forming the pseudostem (Simmonds 1962). Each banana plant produces a single terminal inflorescence. The plant fruits once, then dies and continues to produce successive shoots or suckers from the tuberous rhizome, often referred to as a corm (Karamura et al. 2011; Robinson 1996). The banana inflorescence has three types of flowers that are exposed in sequence. The first to emerge are female flowers (pistillate), followed by neutral or hermaphrodite flowers, and lastly the male flowers (staminate) in the bell (Cheesman 1947). Edible varieties are sterile and parthenocarpic producing fruit without seed, thus requiring propagation by suckers or tissue culture (Robinson 1996; Cheesman 1947; Amah et al. 2021).

Three economically important bacterial wilt diseases affect banana plants. Moko caused by *Ralstonia solanacearum sensu stricto* originating from the Americas (Fegan and Prior 2005), banana Xanthomonas wilt caused by *Xanthomonas vasicola* pv. *musacearum* originating from Africa (Yirgou and Bradbury 1968; Yirgou and Bradbury 1974) and Blood disease caused by *R. syzygii* subsp. *celebesensis* originating from Indonesia (Ray et al. 2021b; Gäumann 1921).

A recent taxonomic revision divided what was previously known as the *R. solanacearum* species complex into three species; *R. solanacearum*, *R. pseudosolanacearum* and *R. syzygii* (Safni et al. 2014). The phenotypically diverse *Ralstonia syzygii* was further separated into three subspecies i) *R. syzygii* subsp. *celebesensis* (Blood disease on banana), ii) *R. syzygii* subsp. *syzygii* (Sumatra disease on clove) and iii) *R. syzygii* subsp. *indonesiensis* (bacterial wilt on a range of solanaceous hosts) (Safni et al. 2014). Bacteria of these species are generally soilborne and move toward plant hosts in wet soil using chemotaxis and flagellar motility (Clough et al. 1997; Yao and Allen 2006; Tans-Kersten et al. 2001). *Ralstonia syzygii* subsp. *syzygii* (Sumatra disease) and *R. syzygii* subsp. *celebesensis* (Blood disease) are exceptions as they lack flagella, reducing their ability to move in soil (Roberts et al. 1990; Eden-Green and Sastraatmadja 1990).

Banana Blood disease causes characteristic symptoms on cultivated and wild *Musa* spp. (Gäumann 1921; Ray et al. 2021b) including wilting, chlorosis and necrosis of leaves, red/brown discolouration of vascular tissues in the pseudostem and peduncle, oozing from male bell followed by necrosis, and rot and discolouration of the fruit pulp (Fig. 1). Transmission of Blood disease is hypothesized to occur predominantly by mechanical transfer of bacteria from an infected oozing male bell to the male bell of a healthy banana plant, followed by infection through open xylem vessels (Ray et al. 2021a; Buddenhagen 2009). The transfer is likely to occur via insects, although bats and birds may also play a role (Mairawita et al. 2015; Leiwakabessy 2003; Hara and Ono 1984; Ray et al. 2021a). Local dispersal is also postulated to be associated with contaminated tools, soil and water (Buddenhagen 2009).

To increase our understanding of the disease cycle of *R. syzygii* subsp. *celebesensis* and mitigate its spread, information regarding its transmission is required. An understanding of

which infected plant parts produce inoculum and become infectious, which plant parts are susceptible to infection, and the mode of transmission between them are vital to manage this disease. The relative roles of tools used for pruning and harvesting, water movement, and root-to-root spread in local disease transmission are unknown. Whilst researchers have postulated that long-distance spread is due to the movement of infected, but visibly healthy suckers from areas where the disease occurs, and from the trade of asymptomatic fruits, this has not been experimentally confirmed.

The overall objective of our research was to elucidate the aetiology of *R. syzygii* subsp. *celebesensis* to scientifically underpin the development of effective disease management practices. Therefore, we sought to address the following specific research questions; 1) Is the ooze and sap exuded from various symptomatic banana plant parts infectious? 2) Does infection occur through fresh cut surfaces exposed by pruning and harvesting practices? 3) Can a knife transmit the bacterium from a diseased to a healthy banana plant? 4) Can sap from a symptomatic inflorescence infect through the roots and cause banana Blood disease? 5) Does *R. syzygii* subsp. *celebesensis* colonize the roots of symptomatic plants? 6) Do roots of symptomatic plants extrude infective bacteria? 7) Can Blood disease be transmitted from the roots of an infected plant to the roots of a healthy plant? 8) Does *R. syzygii* subsp. *celebesensis* pass from the mother plant to the sucker? Addressing these questions will provide a greater understanding of this disease's biology and epidemiology, underpinning the development of management tools that can help prevent spread to currently disease-free areas, and reduce disease incidence where the disease is endemic.

Materials and Methods

Inoculum preparation, re-isolation and identification through PCR

Isolates JR3824 and JR3759, retrieved from cultures stored in microtubes containing sterile water, were previously confirmed as pathogenic and identified as *R. syzygii* subsp. *celebesensis* (Ray et al. 2021b; Rincón-Flórez et al. 2021). Inoculum was prepared just prior to inoculation and adjusted to the required concentration as previously described (Ray et al. 2021b). To prevent cross-contamination during the setting up of experiments, plant maintenance and monitoring, tools and hands were sanitised using 80% ethanol.

Re-isolation and confirmatory PCR-based identification of R. syzygii *subsp.* celebesensis. The symptoms observed and rated as positive for Blood disease in all experiments were confirmed as being caused by *R. syzygii* subsp. *celebesensis* using PCR. To confirm the identity of the causal agent the bacterium was re-isolated as previously described (Ray et al. 2021b) from one symptomatic plant of each banana variety in each treatment in all experiments. DNA was extracted from pure cultures of the isolates and *R. syzygii* subsp. *celebesensis* confirmed as the causal agent of the observed symptoms by PCR using the 121 primer set as previously described (Rincón-Flórez et al. 2021).

Plant material

Tissue cultured banana plants for the potted and field experiments were sourced from Institute Plants Centre, Magelang, Central Java, Indonesia, and Penangkaran Bioteknologi Hortikultura, Pringsewu, Lampung, Indonesia.

Potted plants. Banana plants of variety Kepok 'Kuning' (Kepok) were grown in potting mix in 10-cm and 30-cm diameter pots, and the Cavendish variety was grown in potting mix in 30-cm diameter pots. Potted plants were grown under natural light in a shade house at the

Research Center for Biotechnology Universitas Gadjah Mada, Yogyakarta, Indonesia, with a maximum day temperature of 38°C and a minimum night temperature of 23°C, fertilized (NPK 16-16-16, Multitara™, Medan, Indonesia) and watered regularly.

Field trial. A field trial site was established in Bantul, Special Region of Yogyakarta, Java, Indonesia. A total of 170 Kepok and 190 Cavendish plants were planted as a duplicated block design in October 2018, and irrigated and fertilized for optimal growth. To prevent insect entry and protect the plants from external infection of Blood disease, all plants were bagged soon after the emergence of the bunch with fine mesh cloth bags (SL04-C900 Crownpack™, Kunda Park, QLD, Australia), and closed at the bottom as previously described (Ray et al. 2021a).

Infectious plant parts

To determine if sap and ooze from different infected and symptomatic plant parts can yield bacteria able to initiate disease, sap or ooze from the following plant parts was investigated; i) male bell, ii) cut bunch peduncle, iii) rachis cut to remove the male bell, iv) cut pseudostem, and v) cut fruit. The sap or ooze obtained was used to inoculate potted Kepok plants.

Inoculation of field-grown plants to assess infectivity of plant parts. Field-grown Kepok plants were inoculated with *R. syzygii* subsp. *celebesensis* to express disease symptoms for the collection of ooze and sap for subsequent experimentation. Healthy field-grown plants with bunches covered by fine mesh cloth bags were chosen randomly from plants at the growth stage of a male bell. Inoculum of *R. syzygii* subsp. *celebesensis* isolate JR3824 was prepared as described above and adjusted to approximately 10^7 CFU.mL^{-1}. The bells of four plants were

spray inoculated on the same day with 1.5 mL. To prevent infection from other sources or transmission, the cloth bags were re-secured over the inflorescences after inoculation.

The male bells were visually assessed for ooze production two weeks after inoculation (Fig. 2A and B). The fruit were evaluated 5 weeks after inoculation for disease expression by cutting at least three individual fruit fingers on each bunch using a knife. Plants were considered positive if symptoms of rot and discolouration characteristic of Blood disease were present in the fruit pulp.

Ooze collection from the diseased male bell. Approximately 100 µL of the milky ooze was collected from a symptomatic Kepok bunch two weeks post-inoculation of the male bell. Ooze was collected from around the bract scars, flower cushions, the tips of closed bracts, and the base of the bell using a pipette. The ooze sample was transferred to a 2 mL microtube containing 200 µL of sterile deionized water and mixed using a pipette.

Sap collection from diseased banana parts. A symptomatic Kepok plant was cut down approximately 1 m above ground level for sap sampling 5 weeks post-inoculation. Droplets of sap were collected from fresh cuts made to the bunch peduncle, rachis and the fruit in separate 2 mL microtubes (Fig. 2C). Pseudostem sap was collected by cutting a 'V' shape on the top of the cut pseudostem and allowing the sap to pool. After 5 minutes, the sap was collected using a pipette and transferred to a 2 mL microtube. For each plant part, a volume of 200 µL of sap was mixed with 200 µL of sterile deionized water and mixed using a pipette.

To determine infectivity of each of the sap and ooze samples, Kepok banana plants grown in 10-cm pots at the 5-to 6-leaf-growth stage were inoculated by stem injection approximately 3-cm above the soil. A 1 mL syringe with a 26G needle, was used to inject 100 µL of the diluted

ooze or sap into each test plant slowly. The prepared inoculum was injected within 4 hours of collection. The whole experiment was carried out four times, with the ooze and sap to be used as inoculum collected independently from each of four field-grown plants. Control plants were injected with sterile water. The test Kepok plants were assessed for symptoms weekly for 4 weeks after inoculation. Plants showing symptoms of leaf yellowing, necrosis, wilting and internal vascular discolouration were considered diseased (Fig. 2E and F) and confirmed by PCR as described above.

Infection through exposed plant surfaces

To test if infection can occur during pruning and harvesting practices, cut surfaces on field-grown Kepok and Cavendish bananas were inoculated. The treatments were applied to i) rachis cut to remove the male bell, ii) petioles cut to remove green leaves, iii) pseudostem cut approximately 1 m above the ground to remove the plant, iv) bunch peduncle cut to remove the bunch and v) corm cut to remove a shoot or sucker.

Plant inoculation with R. syzygii *subsp.* celebesensis. Inoculum of isolate JR3824 was prepared as described above and adjusted to approximately 10^7 CFU.mL^{-1}. Plants at the correct growth stage were chosen randomly for the treatments. For the treatments of cut rachis, pseudostem and bunch peduncle, a plant with a bunch and male bell were used to reflect commercial harvesting practices. For the treatments of the cut corm and petiole, the plants used were pre-bud emergence and flowering. For all cut surface treatments, cuts were made between 9:00 am and 12:00 pm and immediately spray-inoculated with 750 µL of the inoculum followed by a pause of 1 minute and a second application of 750 µL. This pause allowed natural sap flow initially rapid to slow from the freshly cut plant surfaces. Following inoculation of the cut rachis, the cloth bunch bag was resealed. The other cut surfaces were

wrapped in a clean cloth bunch bag to protect the inoculation site and to prevent insect transmission of the bacteria to other plants. Each of the five treatments and the controls were replicated at least three times in the Kepok and Cavendish plants, and the controls were bagged to prevent infection. The experiment was fully replicated once at a later date.

Disease assessment. Plants were assessed for disease symptoms 5, 8, 11, 15 and 22 weeks after inoculation. For plants from the cut rachis treatment, at least three fingers were cut from at least three different hands per bunch from each plant after 5 and 8 weeks. Plants were rated as positive when characteristic symptoms of rot and discolouration of the fruit pulp, characteristic of Blood disease, were observed. After 8 weeks, the main pseudostem was cut approximately 1 m above the ground and examined for symptoms of vascular staining. The banana plants from the cut petiole and cut corm treatments were visually assessed for symptoms of leaf wilting, yellowing and necrosis at each time point. The suckers (shoots) growing from the mats of all plant treatments were visually evaluated for symptoms of leaf wilting, yellowing and necrosis at each time point. Suspect shoots expressing symptoms of leaf wilt, yellowing or necrosis were assessed by a cut across the pseudostem. Plants were rated as positive when characteristic vascular staining was present and confirmed by PCR as described above.

Assessments of disease transmission by tools

To determine if tools used for routine pruning of leaves and harvesting practices can transmit the bacteria, experiments were conducted using Kepok and Cavendish plants in 30-cm diameter pots at the 9-to 10-leaf growth stage.

Inoculation of potted plants to establish sources of inoculum for subsequent experiments. Inoculum of isolates JR3824 and JR3759 was prepared as described above and adjusted to approximately 10^8 CFU.mL^{-1}. Two Kepok and two Cavendish plants were each injected slowly with 500 µL of inoculum into the pseudostem. Plants were injected approximately 3-cm above the soil line using a 25G needle. After 12 days of incubation early symptoms of leaf wilt became evident.

Transmission by a knife. Twelve days after inoculation, the inoculum source plants were cut across the stem, at least 20-cm above the soil line for Kepok, and at least 10-cm above the soil line for Cavendish. The sap was allowed to pool through xylem pressure prior to dipping both sides of the tip of a field knife into the sap (Fig. 3A). The field knife was then stabbed through the pseudostem approximately 3-cm above the soil line of the same variety of a healthy banana plant grown in a pot (Fig. 3B). One diseased plant was used to inoculate three healthy plants. The knife was sanitised between each inoculation. The experiment was replicated once for Kepok and Cavendish plants with both isolates. The sap of non-inoculated plants was used for the controls. The whole transmission experiment was repeated on a different day.

Disease assessment. Plants inoculated with a field knife were assessed for disease expression three weeks after inoculation. Plants showing external symptoms of leaf yellowing, necrosis, wilting, and internal vascular discolouration were considered to be diseased (Fig. 3D, F and H). To confirm pathogen presence, isolations were made from discoloured vascular strands from the pseudostem at least 10-cm above the inoculation point and confirmed by PCR as described above.

Assessment of diseased inflorescence sap as root inoculum

The sap from a symptomatic inflorescence was mixed with sterile deionised water and used as inoculum to determine if bacterial transmission can occur from inflorescence sap to the roots of a healthy plant.

Root inoculation. Four field grown Kepok plants inoculated with isolate JR3824 were confirmed symptomatic after 5 weeks. The inoculum was produced from these plants by cutting up the symptomatic fruit, peduncle and rachis tissue and submerging in a beaker containing 200 mL of sterile deionized water. The mixture of approximately 50 % v/v water and plant parts was incubated for 10 minutes, strained to remove plant material and 75 mL of inoculum were poured over unwounded and wounded plant roots of Kepok plants in 10-cm diameter pots at the 5-to 6-leaf stage. Roots were wounded by stabbing a 2-cm wide knife through the roots in 4 different places around the base of the potted Kepok plant prior to inoculation. The Kepok plant pots were wrapped with plastic film (Gladwrap™) for 10 minutes to prevent the inoculum from running out the bottom of the pot. The whole experiment was replicated four times, and the inoculum was prepared each time from a different inoculated field plant. Control plants were treated the same way but flooded with sterile water. The inoculated potted plants were transferred to a shade house, watered regularly, and monitored weekly for 8 weeks.

Disease assessment. Plants expressing symptoms of leaf yellowing, necrosis and wilting were considered to be diseased. Symptomatic plants were cut and examined for internal vascular discolouration and confirmed by PCR as described above.

Root colonization of plants inoculated above ground

To determine if *R. syzygii* subsp. *celebesensis* was present in root tissue of symptomatic plants inoculated above ground level, four Kepok plants growing in 30-cm diameter pots at the 9-to 10-leaf growth stage were inoculated.

Pathogen inoculation, disease assessment and bacterial isolation. Kepok plants were injected with 500 µL of inoculum of isolate JR3824, adjusted to 10^8 CFU.mL^{-1}, into the pseudostem approximately 3-cm above the soil line using a 25G needle. Twelve days after inoculation, three of the fibrous roots attached to the corm were selected from plants showing early wilt symptoms and the soil removed by washing with tap water. The roots were surface sterilized for 1 minute with 1 % sodium hypochlorite (NaOCl), washed with sterile deionized water and then dried on a paper towel. A section of approximately 10 mm was excised from each root, placed into a 2 mL tube containing 1 mL of sterile water and incubated at room temperature for 10 to 20 minutes. Using a 10 µL loop, approximately 40 µL of the solution was streaked onto one edge of the Petri plate containing modified tetrazolium chloride medium (1/2 TZC + C), streaked out across the plate to obtain single colonies, and incubated at approximately 28°C as previously described (Ray et al. 2021b). When slow growing bacterial colonies with red centres and white margins characteristic of *R. syzygii* subsp. *celebesensis* were visible, single-cell cultures were obtained and grown on casamino acid peptone glucose media as previously described (Ray et al. 2021b). One isolate from each plant was selected and confirmed by PCR as described above.

Extrusion of infective bacteria from roots of infected plants

To test if symptomatic plants yield *R. syzygii* subsp. *celebesensis* from their roots into the soil water able to infect and cause disease in healthy plants, four Kepok plants grown in 30-cm diameter pots at the 10-leaf stage were stem inoculated with 500 µL of inoculum from isolate

JR3824 adjusted to approximately 10^8 CFU.mL^{-1}. Four plants were inoculated with water as controls. The inoculated plants showed characteristic symptoms of wilt associated with Blood disease 15 days after incubation. The pots were sealed with gladwrap and watered to just over their water holding capacity, and allowed to incubate for 10 minutes. Subsequently, a volume of 50 mL of soil water was drained from the pot base for use as inoculum. A second batch of inoculum was obtained after wounding the plants roots by stabbing through to the base of the pot four times with a knife of 2-cm diameter to emulate the root damage caused by the commercial practice of removing suckers. The plants were then re-watered just beyond saturation and incubated for 10 minutes, after which 50 mL of soil water was drained from the base of the pot for use as inoculum.

Inoculation of soil water and disease assessment. A volume of 200 µL of the sampled soil water inoculum was subsequently injected 3-cm above the soil line into the stem of Kepok plants grown in 10-cm diameter pot at the 5-to 6-leaf growth stage. The whole experiment was replicated four times. The plants were assessed for symptoms of leaf wilt and yellowing weekly for 5 weeks.

Plant-to-plant transmission

To determine if *R. syzygii* subsp. *celebesensis* can be transmitted from the roots of a diseased plant to the roots of a healthy plant and cause Blood disease, one of two banana plants grown in the same pot were inoculated (Fig. 4). Two Kepok or two Cavendish banana plants were grown in 30-cm pots until they reached the 8-to 9-leaf stage.

Bacterial inoculation. Inoculum of isolates JR3824 and JR3759 were adjusted to approximately 10^8 CFU.mL^{-1}, and 500 µL injected approximately 3-cm above the soil line using a 25G needle

295 into the side of the pseudostem opposite to the second plant in the pot. The injections were
296 performed slowly, and any drops of inoculum formed on the pseudostem were removed with
297 a paper towel. Each isolate and water as a control was applied to one plant of each variety.
298 The experiment included three pots of each isolate/variety combination, and the whole
299 experiment was repeated at a later date. The plants were watered regularly and monitored
300 for symptom development.

301 *Disease assessment on inoculated plants*. Plants were assessed for symptoms of leaf
302 yellowing, necrosis and wilt 3, 6 and 12 weeks after inoculation. Symptoms of leaf wilt and
303 internal vascular discolouration in the non-stem inoculated plants were confirmed as Blood
304 disease by PCR as described above (Fig. 4C).

305 **Blood disease transmission from mother plant to sucker**

306 To determine if *R. syzygii* subsp. *celebesensis* can pass from an infected mother plant to a
307 sucker, field-grown Kepok and Cavendish plants were randomly chosen, and their
308 inflorescence were spray inoculated with 1.5 mL of *R. syzygii* subsp. *celebesensis* isolate
309 JR3824 inoculum adjusted to approximately 10^7 CFU.mL^{-1}. After inoculation, cloth bags were
310 re-secured to prevent infection from other sources and spread of the disease. The experiment
311 consisted of at least 5 inoculated plants for each of Cavendish and Kepok, and at least 3
312 control plants for each banana variety. The whole experiment was repeated at a later date.

313 *Disease assessment*. Disease expression was assessed in the mother plants by cutting at least
314 three fingers per bunch using a knife 5 weeks after inoculation. Plants were confirmed as
315 positive by observing internal symptoms of rot and fruit pulp discolouration characteristic of
316 Blood disease. Plants previously rated as negative at 5 weeks were again inspected at 9 weeks

which also included a check for internal vascular staining in the bunch peduncle and the pseudostem. The suckers of symptomatic mother plants were monitored for disease development 9, 15, 19 and 22 weeks after inoculation. Suckers with symptoms of leaf yellowing, necrosis or wilt were confirmed as symptomatic by examining their pseudostem for characteristic vascular staining and confirmed by PCR as described above.

Results

Infectious plant parts

The male bells of all four field-grown Kepok plants released milky ooze two weeks post-inoculation (Fig. 2A and B), while uninoculated control plants produced no ooze. The milky ooze discharged from the diseased Kepok male bells, when injected into potted Kepok plants, caused disease in 100% of the inoculated plants after 4 weeks (Table 1). The sap from each of the symptomatic cut rachis, bunch peduncle and pseudostem, when injected into potted Kepok plants, also caused disease in 100% of the inoculated plants, whereas the fruit sap caused disease in 50% of the potted Kepok plants (Table 1). Symptoms were first observed in at least 25% of plants from all treatments two weeks after inoculation (Table 1). Control plants remained healthy. The bacteria re-isolated from the symptomatic potted Kepok plants were all confirmed as *R. syzygii* subsp. *celebesensis* using PCR-based diagnostics.

Infection through cut surfaces

Symptoms of Blood disease were expressed after inoculation of all cut surface treatments of Kepok and Cavendish after 11 weeks (Fig. 5A, B, C, D and E). Symptomatic plants were first recorded 5 weeks after inoculation of the cut rachis and corm for both Cavendish and Kepok plants (Fig. 5A and E). In Cavendish, symptoms were first recorded 5 weeks after inoculation

of cut petioles and the pseudostem, and 8 weeks after inoculation of the bunch peduncle (Fig. 5B, C and D). Overall symptom expression was slower in Kepok than in Cavendish (Fig. 5A, B, C, D and E). There was no change in the proportion of symptomatic plants for each treatment from 15 to 22 weeks, except for Kepok inoculated at the bunch peduncle (Fig. 5A, B, C, D and E). In Kepok, 100% of the plants inoculated at the cut rachis, petiole, peduncle, and corm developed symptoms (Fig. 5A, B, D and E). In contrast, approximately 50% of the cut pseudostem inoculated plants developed symptoms after 22 weeks (Fig. 5C). Control plants remained healthy. The bacteria re-isolated from symptomatic tissues were in all cases confirmed to be *R. syzygii* subsp. *celebesensis* by PCR.

Tool transmission

All potted Kepok and Cavendish plants stabbed with a knife dipped in sap from symptomatic plants infected with isolates JR3824 and JR3759 developed symptoms of Blood disease three weeks after inoculation (Fig. 3D, F and H). Control plants remained healthy (Fig. 3E and G). The causal agent was confirmed as *R. syzygii* subsp. *celebesensis* by PCR.

Diseased inflorescence sap as root inoculum

The sap from an infected banana inflorescence mixed with water and applied to wounded and non-wounded roots of potted Kepok bananas caused disease symptoms in 25% of the plants with wounded roots three weeks after inoculation. The causal agent was confirmed as *R. syzygii* subsp. *celebesensis* by PCR. No disease developed in the plants with non-wounded roots, and the controls remained healthy.

Root colonization of symptomatic plants

Ralstonia syzygii subsp. *celebesensis* was isolated from the roots of all potted Kepok plants 12 days after the pseudostem was inoculated through injection. The plants were showing mild symptoms of wilting, leaf droop and chlorosis at that time. The isolated bacteria were confirmed as *R. syzygii* subsp. *celebesensis* using PCR.

Extrusion of infective bacteria from roots of infected plants

Potted Kepok plants remained healthy after being inoculated with the leachate from the root zone of wounded or unwounded roots of Blood disease infected plants. All inoculated plants and the controls remained healthy for 5 weeks.

Plant-to-plant transmission

All stem inoculated Cavendish and Kepok plants developed symptoms of Blood disease three weeks after inoculation (Fig. 4B). Plant-to-plant transmission occurred once, from a Kepok plant inoculated with isolate JR3824 to the second Kepok plant cultivated in the same pot 6 weeks after inoculation (Fig. 4C). The second plant in all other replicates and treatments remained healthy, as well as the control plants (Fig. 4A and B). The bacteria isolated from the symptomatic plant was confirmed as *R. syzygii* subsp. *celebesensis* using PCR.

Blood disease transmission from mother plant to sucker

Following inoculation of the Kepok and Cavendish mother plants with *R. syzygii* subsp. *celebesensis,* 100% of the Cavendish plants had at least one diseased sucker after 15 weeks, and 100% of the Kepok plants had at least one diseased sucker after 22 weeks (Fig. 6 and 7). Symptoms were first observed in the Kepok and Cavendish suckers 9 weeks after inoculation. The suckers expressed symptoms of Blood disease such as wilted, chlorotic, or necrotic leaves which were sometimes water-soaked with black rot, and vascular staining in the pseudostem

(Fig. 7). The suckers that became infected and developed symptoms whilst young collapsed before reaching maturity. All controls remained healthy. Bacterial isolation and PCR confirmed the presence of *R. syzygii* subsp. *celebesensis*.

Discussion

The results show that the ooze from the male bell and sap from a cut symptomatic bunch peduncle, pseudostem, fruit, and the rachis was infective. The cut surfaces of a bunch peduncle, petiole, corm, and the rachis acted as infection courts for *R. syzygii* subsp. *celebesensis* and the bacterium was easily transmitted by cutting tools. The roots of a symptomatic plant were shown to become colonized with the bacterium, and that transmission can occur from the roots of an infected plant to the roots of a healthy plant, and that infected plants transmit the bacterium to their suckers.

Our findings that the sap and ooze obtained from various symptomatic banana plant parts are infective agree with the conclusion that Blood disease becomes systemic (Hadiwiyono 2011). *Ralstonia syzygii* subsp. *celebesensis* may reach high concentrations in the xylem sap. Although this was not quantified, it may be comparable to *R. solanacearum* in tomato, which can reach a bacterial concentration of 10^9 CFU.cm^{-1} in stem tissue (Huang and Allen 2000). The likely high concentrations of the bacterium in xylem sap and ooze, in association with findings that a bacterial concentration as low as 10^2 CFU.mL^{-1} of *R. syzygii subsp. celebesensis* can initiate Blood disease (Ray et al. 2021a), supports the hypothesis that this bacterium is highly infectious.

This study demonstrates that Blood disease is readily tool transmissible from a diseased plant to a healthy banana plant using a field knife. Research on Moko disease, caused by *R.*

404 *solanacearum*, has shown a high incidence of tool transmission in banana plants, for example,
405 field experiments involving routine commercial banana pruning activities showed
406 transmission rates for 85% for de-suckering and 100% for de-belling and de-leafing (Sequeira
407 1958). For Xanthomonas wilt (*Xanthomonas vasicola* pv. *musacearum*) tool transmission rates
408 were 67% through cut green leaves, 100% for cut pseudostem and de-belling and 90% for de-
409 suckering (Addis et al. 2010). Thus, the transmissibility of Blood disease is of the same order
410 as that shown for both Moko disease and Xanthomonas wilt.

411 We show that wounded roots of the banana plant can act as infection courts for Blood
412 disease. This aligns with earlier research which demonstrated infection through the roots of
413 wounded potted banana plants and no infection through non-wounded roots inoculated with
414 *R. syzygii* subsp. *celebesensis* at 10^8 CFU.mL^{-1} (Baharuddin 1994). In our study, disease
415 developed in only one out of the four plants with wounded-roots inoculated with
416 inflorescence sap mixed with water, indicating that although infection can occur through the
417 roots that it may be an infrequent occurrence under natural conditions. *Ralstonia*
418 *solanacearum* readily infects through plant roots. Its ability to move toward the roots of a
419 plant using flagellar motility is an important aspect of soil-borne pathogenicity (Tans-Kersten
420 et al. 2001). Unlike *R. solanacearum*, *R. syzygii* subsp. *celebesensis* lacks flagella (Eden-Green
421 and Sastraatmadja 1990) and thus relies on passive contact with roots reducing its likelihood
422 of finding a natural opening or wound for infection. The result is that *R. syzygii* subsp.
423 *celebesensis* may be less efficient at infecting through roots when compared to the other
424 *Ralstonia* species.

425 Water drained from the roots of wounded and unwounded symptomatic Blood disease
426 infected banana plants when used as inoculum, did not result in expression of Blood disease.

In contrast, similar experiments carried out on tomato plants with early wilt symptoms after inoculation with *R. solanacearum* demonstrated that the soil water drained from the base of these pots contained *R. solanacearum* at approximately 10^8 CFU.mL^{-1} (Kelman 1965). The concentration of *R. syzygii* subsp. *celebesensis* was not measured in the soil water during this study but, given that a volume of 500 uL of inoculum at 10^2 CFU.mL^{-1} can cause disease when injected into potted plants (Ray et al. 2021a), it may be assumed there was a negligible amount of bacteria present in the soil water under our experimental conditions. It is possible that the roots of plants infected with *R. syzygii subsp. celebesensis* may release fewer bacteria into the soil water than Moko caused by *R. solanacearum*. Further experimentation using different time frames and incubation conditions are required to investigate this hypothesis.

We have shown that the roots of a banana plant become colonized with the bacterium following stem inoculation with *R. syzygii* subsp. *celebesensis,* and that transmission can occur from the roots of a diseased plant to the roots of a healthy plant grown in close proximity. *Ralstonia solanacearum* transmission rates of close to 100% have been reported for paired tomato and paired tobacco plants (Kelman 1965). Our pot experiments demonstrated root-to-root transmission of *R. syzygii* subsp. *celebesensis* but at a low frequency. This apparent difference in root transmissibility between other *Ralstonia* spp. and *R. syzygii* subsp. *celebesensis* may highlight a biological difference between the species and could be related to the presence or absence of flagella.

Our results demonstrate that transmission of *R. syzygii* subsp. *celebesensis* can occur through water containing the sap and ooze from symptomatic plant parts and infect through the wounded roots of a healthy banana plant causing Blood disease. However, this study does not provide evidence concerning the overall role or relative importance of water in the

transmission and dispersal of Blood disease in the field. The combined results of our study; low propensity to infect roots, infrequent plant-to-plant transmission and apparent low propensity to extrude infective bacteria from roots imply that plant-to-plant transmission through water may not be an important transmission mechanism for Blood disease. Unlike other *Ralstonia* species including Moko disease where water plays a significant role in transmission and dispersal through flooding and contamination of watercourses (Tomlinson et al. 2009; Wenneker et al. 1999; Coelho Netto et al. 2003; Sequeira and Averre 1961). Our data suggest that *R. syzygii* subsp. *celebesensis* is not efficient at infecting through roots and that infected banana roots appear to have a low propensity to extrude infective bacteria. These distinct biological differences between *R. syzygii* subsp. *celebesensis* and other vascular wilts caused by *Ralstonia* spp. imply that Blood disease may have a somewhat different disease cycle that warrants further investigation.

Soil containing *Ralstonia* spp. plays a significant role in the transmission of other bacterial wilt diseases. For example, soil transmission is important in the disease cycle of *R. solanacearum sensu stricto* causative agent of Moko, as the soil remains infective for a period of 6 to 12 months after removal of diseased bananas (Sequeira 1958; Stover 1972). Research conducted on Moko also demonstrated that bacteria present in infested soil were able to infect healthy banana plants through wounds created during sucker removal (Sequeira 1958). Yet, there is no data available concerning the role of soil in the transmission of *R. syzygii* subsp. *celebesensis* and the ability of the bacterium to remain in the soil and be transmitted to healthy plants.

Our results showing that Blood disease moves from the mother plants to the suckers, causing disease in Kepok and Cavendish, supports earlier studies (Gäumann 1921; Stover and

473 Espinoza 1992). The suckers in our study expressed symptoms after a period of at least five
474 weeks following the inoculation of parent plants and over time all banana mats developed
475 diseased suckers. Our finding that diseased plants eventually yield symptomatic suckers
476 supports the hypothesis that long-distance dispersal of *R. syzygii* subsp. *celebesensis* can
477 occur by the movement of asymptomatic contaminated suckers.

478 An understanding of the biology and disease cycle of Blood disease aids the development of
479 effective control measures. Our results show that local dispersal of Blood disease can take
480 place through mechanical transmission by tools. We also demonstrate that symptomatic
481 plant parts are infectious and cut surfaces act as infection courts supporting the hypothesis
482 that any means including insects, tools, bats, birds or animals that can transfer the bacteria.
483 We recently experimentally confirmed that infection takes place through open xylem vessels
484 located at fresh-cut plant parts, the female flowers, and are exposed when bracts and male
485 flowers abscise (Ray et al. 2021a). In our experimental field site in Java, Indonesia, we
486 effectively excluded insect transmission by the use of cloth bags sealed above and below the
487 bunch, and tool transmission by sterilisation of implements which can be used as disease
488 control strategies. From a smallholder perspective, changes to the use of cutting tools are
489 also required to prevent spread of bacteria from diseased to healthy banana plants. The use
490 of disease-free planting material is also very important to reduce local spread while also
491 reducing long-distance dispersal.

492 The ongoing geographic expansion of Blood disease across the Indonesian archipelago and
493 more recently to Malaysia (Ray et al. 2021b; Teng et al. 2016) highlights the urgent need for
494 the implementation of effective disease management strategies and extension of these
495 across the region. Control measures such as tool sanitation, early detection and destruction

of diseased mats, minimal pruning, de-belling without cutting the rachis, bagging inflorescences at emergence using sealed cloth bags, and access to clean planting materials need to be considered. Heightened awareness of Blood disease and its control measures by farmers and other stakeholders is also critical to prevent further geographic spread and prevent or reduce crop losses associated with this destructive disease.

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578 emend the descriptions of *Ralstonia solanacearum* and *Ralstonia syzygii* and reclassify
579 current *R. syzygii* strains as *Ralstonia syzygii* subsp. *syzygii* subsp. nov., *R.*
580 *solanacearum* phylotype IV strains as *Ralstonia syzygii* subsp. *indonesiensis* subsp.
581 nov., banana blood disease bacterium strains as *Ralstonia syzygii* subsp. *celebesensis*
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Tables

Table 1. Ability of ooze and sap from diseased banana plant parts to cause symptoms of Blood disease when injected into a potted Kepok plant, monitored for four weeks after inoculation. *Ralstonia syzygii* subsp. *celebesensis* isolate JR3824 utilized for experiments.

Inoculum source	Week 2[a]	Week 3[a]	Week 4[a]
Male bell ooze	3/4	3/4	4/4
Rachis sap	4/4	4/4	4/4
Bunch peduncle sap	2/4	3/4	4/4
Pseudostem sap	3/4	3/4	4/4
Fruit sap	1/4	2/4	2/4
Control	0/4	0/4	0/4

[a] Number of symptomatic plants / total number of plants inoculated.

Figures

Fig. 1. Symptoms of Blood disease in Cavendish caused by *Ralstonia syzygii* subsp. *celebesensis* isolate JR3824. **A,** Oozing of the male bell. **B,** Desiccation of the male bell. **C,** Vascular staining in the pseudostem. **D,** Vascular staining in the bunch peduncle. **E,** Wilt, chlorosis, scorch and necrosis of leaves. **F, G,** Pulp rot and internal discoloration of green banana fruits.

Fig. 2. **A,** and **B,** Oozing from bract scars, flower cushions, and the male bell of diseased Kepok plants two weeks after inoculation. **C,** Collection of sap from symptomatic banana fruit for inoculation. Sap and ooze infectivity assessment through inoculation of potted Kepok with; **D,** water as a control; and sap from the symptomatic **E,** pseudostem, and **F,** the rachis. *Ralstonia syzygii* subsp. *celebesensis* isolate JR3824 utilized for experiments.

Fig. 3. Tool transmission of *Ralstonia syzygii* subsp. *celebesensis* with isolates JR3824 and JR3759. **A,** Knife dipped in the sap of a diseased plant. **B,** Stem inoculation with a sap dipped knife. **C,** Asymptomatic pseudostem of a control plant. **D,** Vascular staining in pseudostem of an inoculated plant after 3 weeks. **E,** Cavendish control. **F,** Inoculated Cavendish plant showing symptoms of wilting after 3 weeks. **G,** Kepok control. **H,** Inoculated Kepok plant showing symptoms of wilting after 3 weeks.

Fig. 4. Plant-to-plant transmission of *Ralstonia syzygii* subsp. *celebesensis* in the Kepok banana variety. **A,** Control, two healthy plants. **B,** Only the stem inoculated on the left with isolate JR3824 developed symptoms of Blood disease. **C,** Inoculated with isolate JR3824 and non-inoculated plants showing symptoms of Blood disease. Arrow denotes inoculated plant.

671 **Fig. 5.** Percentage of Kepok and Cavendish banana plants expressing symptoms of Blood
672 disease over time following inoculation of different fresh-cut plant parts with *Ralstonia syzygii*
673 subsp. *celebesensis* isolate JR3824.

674 **Fig. 6.** Percentage of banana plants with suckers developing Blood disease symptoms
675 following inoculation of the mother plants of field-grown Kepok and Cavendish banana
676 varieties with *Ralstonia syzygii* subsp. *celebesensis* isolate JR3824.

677 **Fig. 7.** Disease transmission from mother plants inoculated with *Ralstonia syzygii* subsp.
678 *celebesensis* isolate JR3824 to suckers. **A,** Kepok banana with diseased suckers. **B,** Vascular
679 staining in diseased Kepok sucker. **C,** Cavendish with a diseased sucker. Arrows denote
680 diseased suckers.

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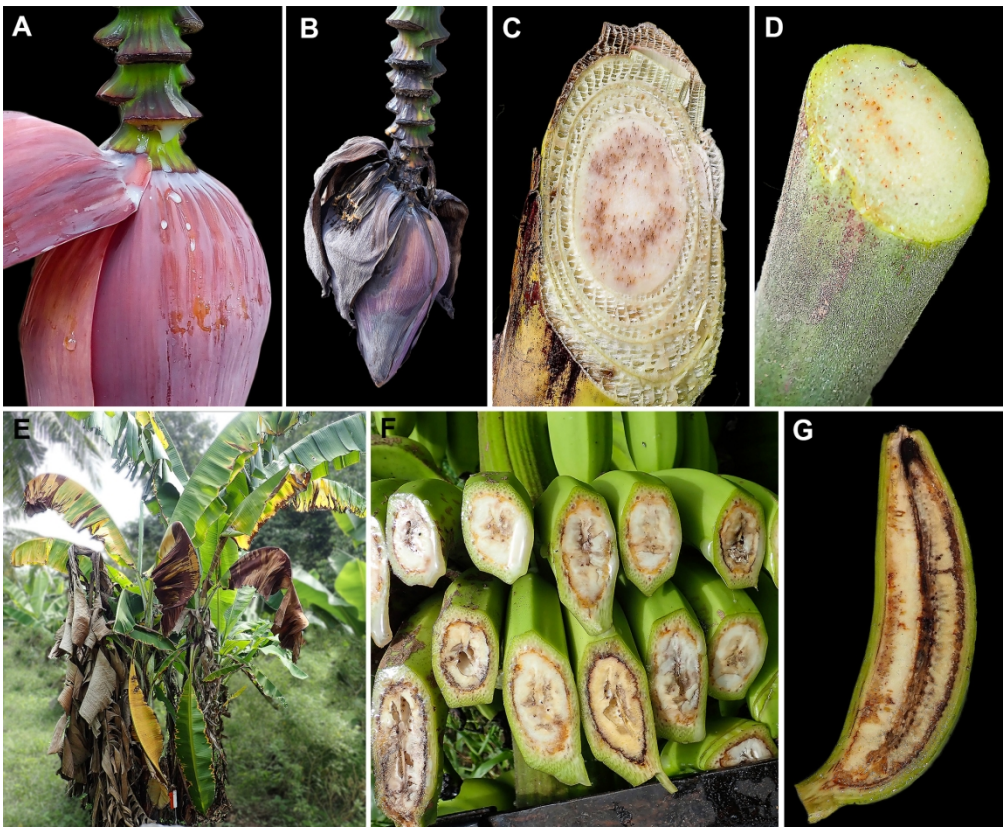

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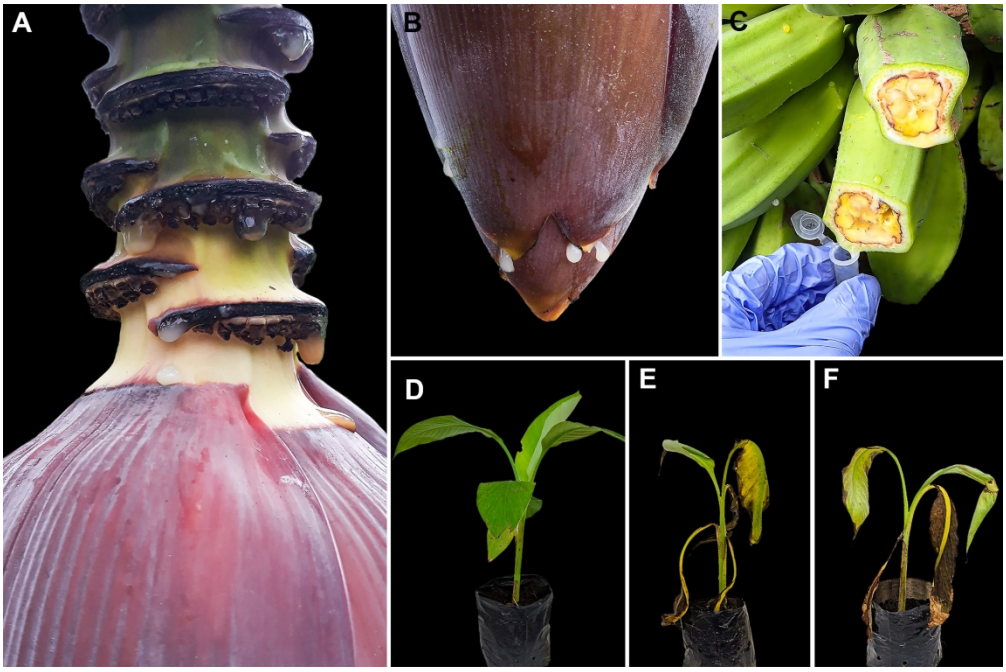

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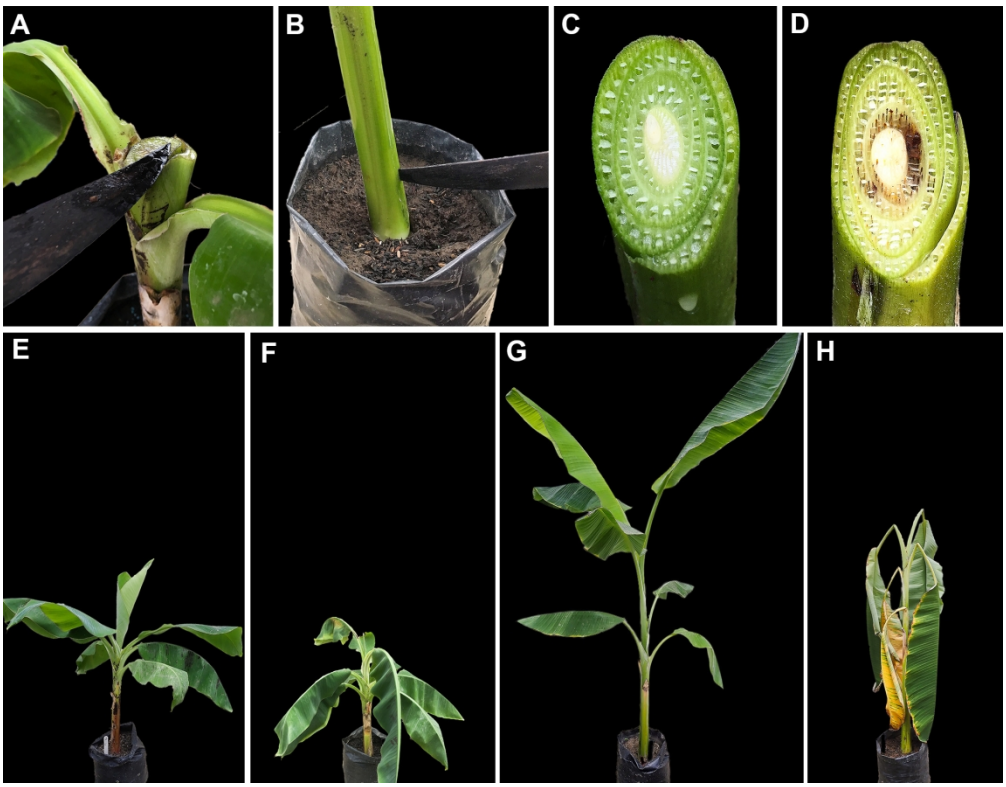

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177x138mm (300 x 300 DPI)

Fig. 4. Plant-to-plant transmission of *Ralstonia syzygii* subsp. *celebesensis* in the Kepok banana variety. **A,** Control, two healthy plants. **B,** Only the stem inoculated on the left with isolate JR3824 developed symptoms of Blood disease. **C,** Inoculated with isolate JR3824 and non-inoculated plants showing symptoms of Blood disease. Arrow denotes inoculated plant.

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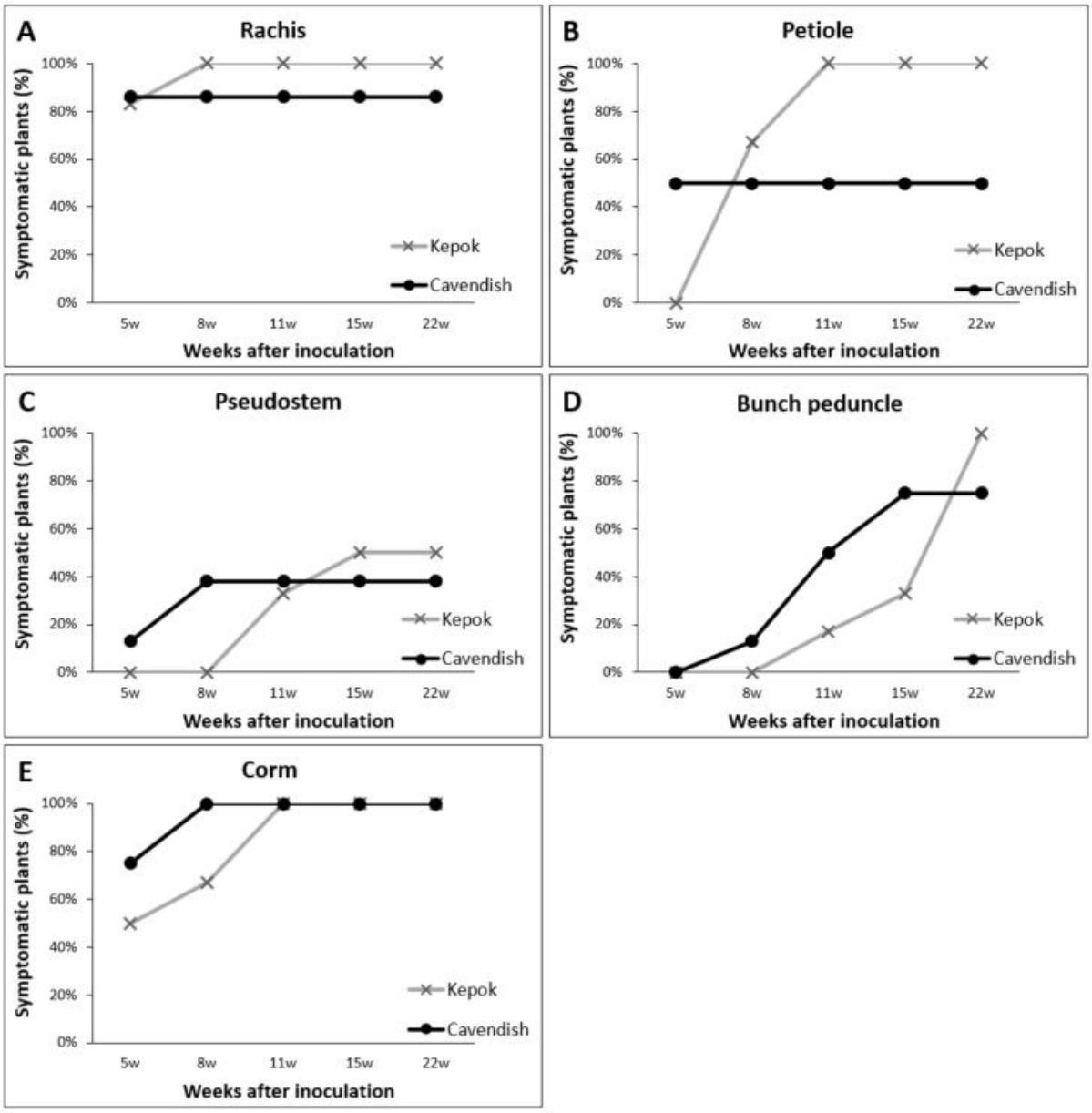

Fig. 5. Percentage of Kepok and Cavendish banana plants expressing symptoms of Blood disease over time following inoculation of different fresh-cut plant parts with *Ralstonia syzygii* subsp. *celebesensis.*

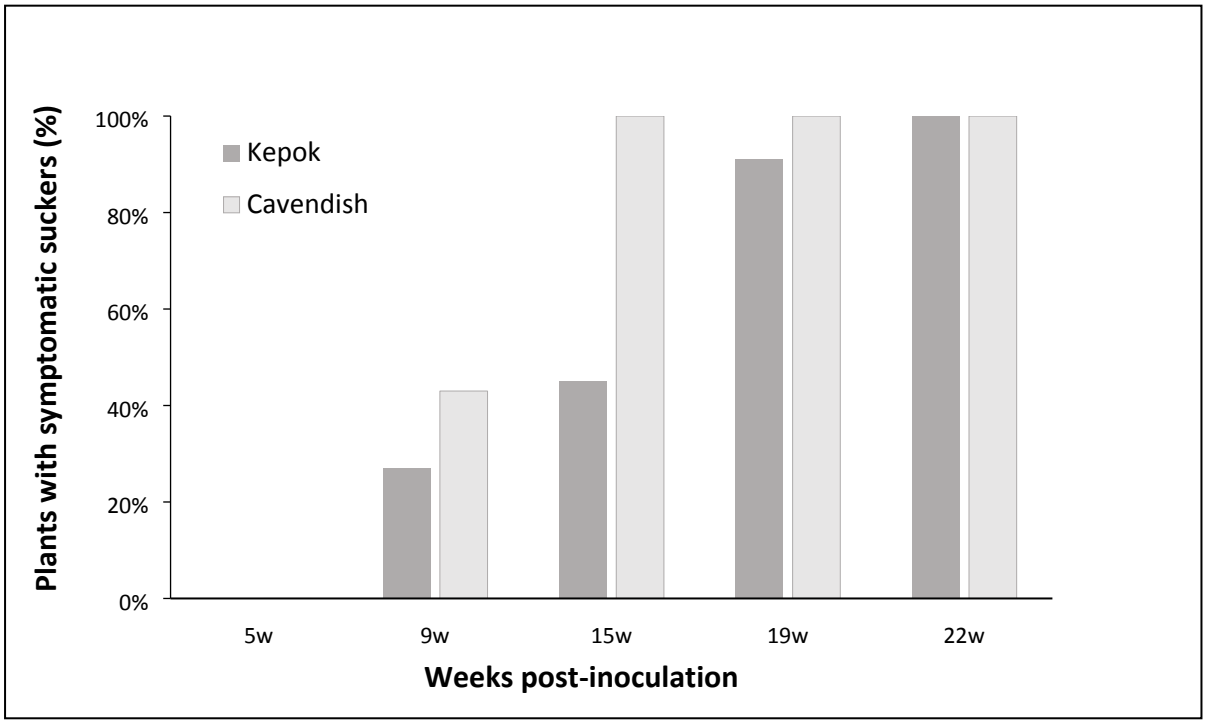

Fig. 6. Percentage of banana plants with suckers developing Blood disease symptoms following inoculation of the mother plants of field-grown Kepok and Cavendish banana varieties with *Ralstonia syzygii* subsp. *celebesensis.*

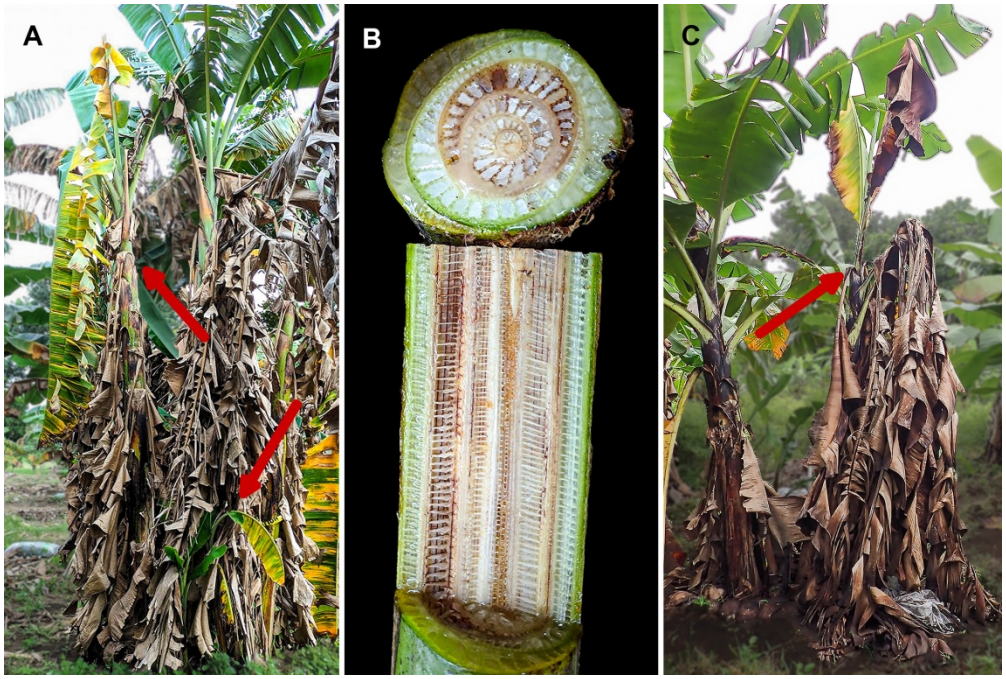

Fig. 7. Disease transmission from mother plants inoculated with *Ralstonia syzygii* subsp. *celebesensis* isolate JR3824 to suckers. **A,** Kepok banana with diseased suckers. **B,** Vascular staining in diseased Kepok sucker. **C,** Cavendish with a diseased sucker. Arrows denote diseased suckers.

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