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Research Article

Motility and Biofilm of *Ralstonia syzygii* subsp. *celebesensis*, the Causative Agent of Banana Blood Disease

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ABSTRACT

Blood disease in bananas is caused by *Ralstonia syzygii* subsp. *celebesensis*. This disease is a significant phytopathological concern, leading to deterioration of the plant's xylem tissue. Symptoms observed in infected plants typically begin from the shoot and banana inflorescence and progressively spread to other parts of the plant. The spread of these symptoms may result from the motility of *R. syzygii* subsp. *celebesensis* within the vascular system. This study aims to investigate the bacterial motility and biofilm formation ability, both contributing to the blockage of water and nutrient flow in the plant. The research methods included bacterial cultivation, molecular detection, motility assays (swimming and swarming tests), as well as biofilm and pellicle formation assay. The results indicated that *R. syzygii* subsp. *celebesensis* exhibited sliding motility but did not demonstrate swarming ability. Additionally, the bacterium was capable of forming both biofilm structures and pellicle layers. These findings provide insights into the role of motility types and colony structure formation in the virulence of *R. syzygii* subsp. *celebesensis*, potentially influencing the infection process in banana plants. Furthermore, this study's results are expected to contribute to developing improved disease management strategies.

Keywords: banana; biofilm; motility; *Ralstonia syzygii* subsp. *celebesensis*

INTRODUCTION

Blood disease bacterium (BDB) is one of the significant diseases affecting banana crops. According to Montong and Salaki (2019), the incidence of banana blood disease in South Minahasa region in 2005 ranged from 30% to 40%, and the estimated national banana production loss due to the disease was approximately 36%. As reported by Ray *et al.* (2021), this disease can cause yield losses ranging from 75% to 100% in banana plantations in the Sumba region, Indonesia. The disease is caused by infection with the pathogenic bacterium *Ralstonia syzygii* subsp. *celebesensis* invades the plant's xylem tissues (Buddenhagen, 2009). This bacterium is a Gram-negative, non-motile, rod-shaped microorganism measuring approximately 0.8×2.5 µm. It is aerobic and tests positive for both catalase and oxidase activity. Colonies generally appear round, mucoid, non-fluid, and small in size (0.5−2 mm) after being incubated at 28 °C for 4-5 days on CPG medium (Safni *et al.*, 2014). Bacterial colonies exhibit smooth edges and a deep red coloration at the center when grown on media containing tetrazolium chloride (TZC) (Prakoso *et al.*, 2022).

The spread of *R. syzygii* subsp. *celebesensis* is predominantly mechanical, occurring from infected to healthy plants through wounds or natural openings (Ray *et al.*, 2022a). Pathogen transmission in the field can occur via various media, including soil, water, agricultural tools, planting materials, and vectors such as insects, bats, and birds that feed on the male flowers of infected plants and subsequently transmit the bacteria to healthy plants (Ray *et al.*, 2022b). Sahetapy *et al.* (2020) identified insects from the Drosophilidae, Tephritidae, and Muscidae families as potential pathogen vectors. According to Rincón-Flórez *et al.* (2022), the bacteria likely move from the banana's male flower to the lower parts of the

plant via fruits and stems, gradually spreading to the corm. Once in the corm, the bacteria invade the vascular tissues, spreading to the leaves and eventually causing characteristic symptoms such as leaf yellowing (Buddenhagen, 2009).

The downward movement of *R. syzygii* subsp. *celebesensis* within banana plant tissues, from the upper parts to the corm, is facilitated by bacterial motility, which refers to the movement of cells driven by various mechanisms (Madigan *et al.*, 2010). Palma *et al.* (2022) highlighted that motility enables bacteria to move away from unfavorable conditions and locate nutrient sources. Also, motility is crucial for interacting with microorganisms and their hosts, particularly during colonization and biofilm formation.

Bacteria can migrate across surfaces through passive mechanisms, such as sliding, or active mechanisms influenced by structures like flagella and pili. Active movements include swimming, swarming, twitching, and gliding (Hölscher & Kovács, 2017). According to Zegadło *et al.* (2023), all of these motility mechanisms facilitate bacterial movement and contribute to infection processes. Swimming motility enables bacteria to disperse from persister cells within biofilm microcolonies and colonize new tissues. Twitching motility assists bacteria in penetrating host tissues during infection. Sliding motility enables cocci—typically considered non-motile—to travel across surfaces. During swarming, bacteria exhibit enhanced resistance to antimicrobial agents. Movement is driven by molecular motors that form focal adhesion complexes within the bacterial membrane, creating a wave-like force that propels bacteria lacking appendages.

Infection symptoms caused by *R. syzygii* subsp. *celebesensis* includes discoloration and wrinkling of male flower buds and stalks, yellowing of young banana leaves followed by necrosis and drying mortality, reddish discoloration of fruit pulp, and the release of reddish-brown bacterial masses upon cutting infected tissues (Ho *et al.*, 2023). These bacterial masses result from colonization of the banana xylem by *R. syzygii* subsp. *celebesensis*, forming biofilm structures that obstruct water and nutrient flow. Pathogenic bacteria are known to form biofilm-like aggregates in xylem vessels, on root and leaf surfaces, but not in intercellular spaces (Mansfield *et al.*, 2012).

Bacterial cells can exist in planktonic form or as biofilms, which are multicellular structures. Despite architectural and compositional variations, biofilms comprise living and dead bacterial cells embedded in a matrix of exopolysaccharides (EPS), lipids, proteins, extracellular DNA, and lysis byproducts (Tran *et al.*, 2016). Biofilm formation is closely linked to bacterial motility, which plays a critical role in the initiation, development, and growth of biofilms by facilitating movement toward surfaces and aggregation. Motility influences biofilm architecture and stability, enabling nutrient access and intercellular communication (O'Toole & Kolter, 1998a).

Once biofilms are established, most bacterial cells become non-motile, forming an integral part of the biofilm, while some remain planktonic, moving through xylem tissues driven by sap flow. Bacteria such as *Ralstonia solanacearum* and *Xanthomonas* utilize flagella for swimming motility within sap flow (Tans-Kersten *et al.*, 2001; Lee *et al.*, 2003; Navitasari *et al.*, 2020). However, *Xylella fastidiosa*, which lacks flagella, relies on type IV pili (TFP) for movement in xylem tissues (De La Fuente *et al.*, 2007).

Flagella and TFP are essential for motility, biofilm formation, pathogenicity, and bacterial virulence. According to Conrad *et al.* (2011), TFP mediates two types of surface motility: crawling, where bacteria move longitudinally with high directional persistence, and upright walking, which allows rapid environmental exploration. Flagella enable additional mechanisms, such as swimming and surface anchoring, often preceding detachment. The interplay between these appendages influences motility during division and detachment, significantly impacting initial biofilm formation. TFP also contributes to uniform biofilm morphology by facilitating vertical orientation and detachment from surfaces.

For pathogenic bacteria, biofilms provide protection against antimicrobial agents, antibiotics, host defense compounds, and toxins released by infected plants (Bogino *et al.*, 2013). Biofilm structures play a crucial role in pathogenicity and virulence by colonizing xylem vessels and obstructing sap flow, leading to wilting and vascular discoloration in host plants (Remenant *et al.*, 2011; Mori *et al.*, 2016). However, dense biofilm structures can restrict sap flow, limiting nutrient availability. At this stage, biofilm-associated cells express high levels of viru-

lence by producing key pathogenicity factors such as plant cell wall-degrading enzymes (PCWDEs) and type III secretion system (T3SS) effectors (Mina *et al.*, 2019).

Motility and biofilm formation are critical for the pathogenicity of *R. syzygii* subsp. *celebesensis* is causing severe damage and losses to banana crops. However, this bacterium's specific motility mechanisms and biofilm formation remain unexplored. This study aims to identify the motility types employed by *R. syzygii* subsp. *celebesensis* and its capacity to form biofilm structures.

MATERIALS AND METHODS

Cultivation of *Ralstonia syzygii* subsp. *celebesensis*

The bacterial sample used was UGMSS_Db01, which is a collection of isolates from the Laboratory of Plant Pathology, Faculty of Agriculture, Universitas Gadjah Mada. The isolate of *R. syzygii* subsp. *celebesensis* was purified on CPG (Casamino acid-Peptone-Glucose) agar medium supplemented with TZC (tetrazolium chloride). The CPG medium is composed of peptone (10 g/L), casein hydrolysate (1 g/L), glucose (5 g/L), and agar (20 g/L). The bacteria were incubated at room temperature for 4−7 days (Prakoso *et al.*, 2020).

Motility Assays

Motility assays were performed on semi-solid media containing 0.2% agar for swimming and sliding assay and 0.8% agar for swarming assay in Petri dishes. This test was conducted based on the method by Joko *et al.* (2007a) developed with slight modifications. Modifications made include the concentration and type of media used, as well as the observation period. A bacterial suspension prepared using sterile distilled water was inoculated by placing 1 µl of the suspension in the center of the Petri dish. Each experiment was conducted with three replications. The motility was observed every 4 hours for a total duration of 68 hours. The parameter in this assay was determined by whether bacterial spreading occurs on the surface of the semi-solid medium within the specified observation period.

Biofilm Assay

Biofilm formation of *R. syzygii* subsp. *celebesensis* was observed following the method developed by O'Toole & Kolter (1998b) with slight modifications. This experiment was conducted with three replications. The bacterial isolate cultured on CPG agar medium for 72 hours (OD600 at 0.8) was diluted in liquid CPG medium (1:10). Then, 100 µl of the bacterial suspension (1×10^5 CFU/ml) was added to each well of a microtiter plate and incubated for 10 hours at 27 °C. After incubation, the suspension was removed, and the wells were air-dried for 15 minutes. Subsequently, 200 µl of 1% crystal violet solution was added to each well for bacterial cell staining. The microtiter plate was then incubated at room temperature for 20 minutes and rinsed thrice with sterile water. The formed biofilm appeared as a purple ring along the walls of the microtiter wells.

Pellicle Assay

Pellicle formation of *R. syzygii* subsp. *celebesensis* was observed following the method developed by Joko *et al.* (2024) with slight modifications. This experiment was conducted with three replications. The bacterial isolate was suspended and adjusted to a 1×10^5 CFU/ml density. A total of 10 ml of liquid CPG medium was added to a test tube, followed by 2 µl of the bacterial suspension, and mixed thoroughly. The medium containing bacteria was incubated at room temperature and observed for 10 days. The pellicle formed was visible as a bacterial colony layer on the surface of the CPG medium.

RESULTS AND DISCUSSION

Cultivation of *Ralstonia syzygii* subsp. *celebesensis*

R. syzygii subsp. *celebesensis* (UGMSS_Db01) was incubated on CPG agar medium supplemented with tetrazolium chloride (TZC) for five days at 26 °C. The results showed bacterial colony morphology characterized by round, convex colonies with smooth edges. The colonies displayed a red center surrounded by white edges (Figure 1).

Based on these findings, *R. syzygii* subsp. *celebesensis* is classified as a slow-growing bacterium, with a growth period ranging from 4 to 5 days. Additionally, the colonies were sticky when transferred to a new medium using an inoculation loop. These observations align with those of Imas *et al.* (2018), who described the colonies of *R. syzygii* subsp. *celebesensis* as round, red with white edges, and con-

vex when cultured on media containing TZC. Similarly, Edy *et al.* (2011) and Ho *et al.* (2023) reported that the colonies of *R. syzygii* subsp. *celebesensis* exhibit a sticky texture and slow growth and become clearly visible on the fourth to fifth day of incubation.

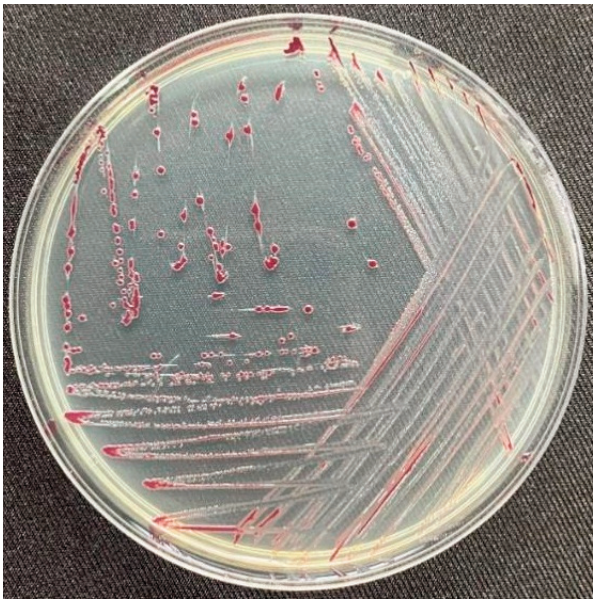

Figure 1. Isolate of *Ralstonia syzygii* subsp. *celebesensis* on Casamino acid-Peptone-Glucose (CPG) agar medium containing tetrazolium chloride (TZC) after incubation for 5 days.

Motility Assays

Swimming and Sliding Motility

Observations indicated that *R. syzygii* subsp. *celebesensis* appeared capable of moving and spreading across the surface of semi-solid media (0.2% agar), filling the petri dish within 68 hours (Figure 2). Notably, this bacterium lacks flagella and is reported to be non-motile in publications by Aisyah *et al.* (2018) and Ho *et al.* (2023). Therefore, further investigation is required to understand how this bacterium can spread and occupy the petri dish.

R. syzygii subsp. *celebesensis* is known to infect the xylem tissue of banana plants, where it proliferates and colonizes host tissues by forming biofilm structures composed of large amounts of exopolysaccharides (EPS) (Mori *et al.*, 2016). According to Hölscher and Kovács (2017), sliding motility is classified into three groups based on the factors driving bacterial movement. Group I includes bacteria that require only the force generated by cell division and secreted surfactants. Group II involves bacteria that depend on additional secreted components such as EPS. Group III comprises bacteria that require growth and other factors but do not utilize surfactants.

Based on this characteristic, *R. syzygii* subsp. *celebesensis* may be categorized within Group II of sliding motility, as defined by Hölscher and Kovács (2017). This classification is supported by its EPS production and the colony morphology observed in this study. According to Hölscher and Kovács (2017), bacteria in Group II exhibit dendritic and planar colony shapes during sliding motility assays.

Research by Matsuyama *et al.* (1995) on the motility of *Serratia marcescens* demonstrated that non-flagellated bacteria can spread on the surface of low-agar media. This type of movement, known as passive motility, is attributed to bacterial cell division activity (Hölscher & Kovács, 2017). Based on Palma *et al.* (2022), non-motile species may exhibit the ability to spread on semi-solid media, although at a lower rate and slower pace compared to species with high motility. Based on a motility study conducted by Martinez *et al.* (1999) using *Mycobacterium smegmatis*, a known non-motile mycobacteria, agarose concentrations of 0.2% or higher were sufficient to allow bacterial cell spreading across a wide surface area, eventually surrounding the inoculation site with a circular halo, as observed in the present study. The diameter of the halo was inversely proportional to the agarose concentration, indicating that the moisture level of the medium is a critical parameter influencing surface spreading. While the exact factors enabling *R. syzygii* subsp. *celebesensis* to spread on semi-solid media remain unclear, the absence of flagella suggests that the observed spreading in semi-solid media assays may occur as a result of sliding motility.

Swarming Motility

Observations showed that *R. syzygii* subsp. *celebesensis* did not exhibit spreading behavior during swarming motility assays. This was indicated by the lack of a significant increase in colony diameter over the 68-hour observation period (Figure 3). This result may be attributed to the absence of flagella in *R. syzygii* subsp. *celebesensis*, as confirmed microscopically using electron microscopy (Aisyah

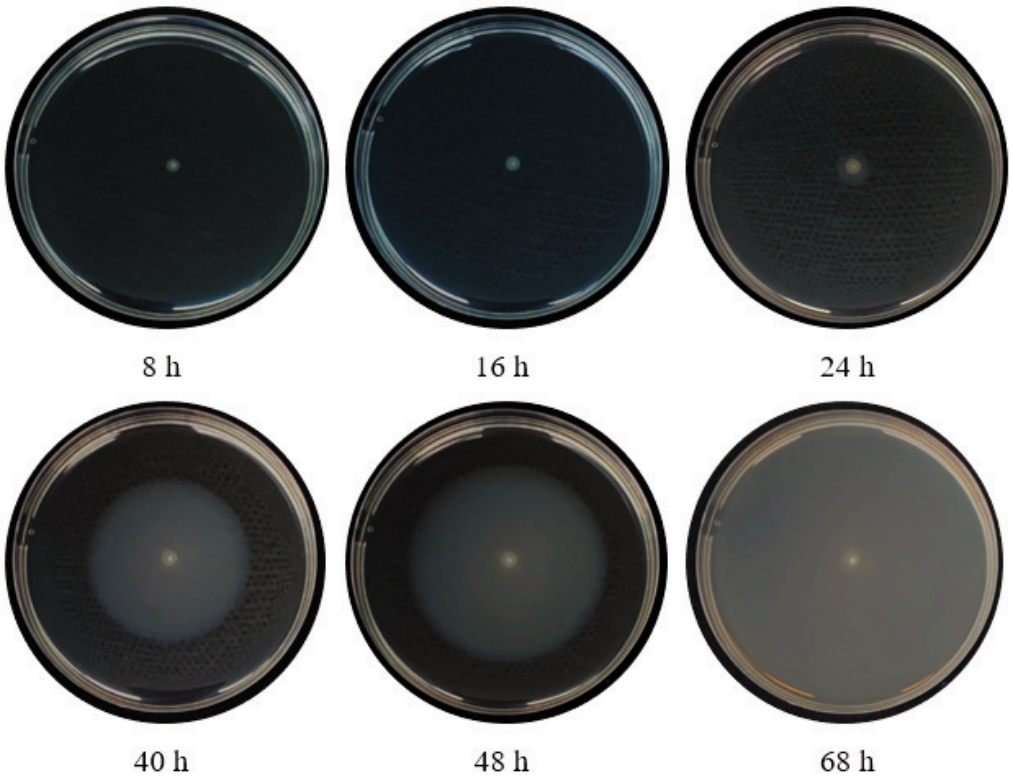

Figure 2. Swimming motility test results of *Ralstonia syzygii* subsp. *celebesensis* after 68 hours of observation, showing the development of the bacterial colony diameter that fills the Petri dish.

et al., 2018). Flagella are the primary structures enabling movement during swarming motility on solid media, suggesting that the bacterium is incapable of swarming motility.

These findings are consistent with research by Kearns (2010) on *Bacillus subtilis* 3610 mutants, which demonstrated that non-swarming mutants formed colonies confined to the central center of the agar. During overextended incubation periods, the colony diameter of non-swarming bacteria increased due to contributions from sliding motility.

Swarming motility is a rapid, multicellular form of bacterial movement across the surface of a medium, driven by rotating flagella (Henrichsen, 1972; Rashid & Kornberg, 2000). This type of motility can involve cellular differentiation, where bacteria develop elongated, hyper-flagellated phenotypes (Kearns, 2010). The presence of flagella is crucial for swarming motility. According to Taguchi and Ichinose (2011), the degree to which flagella and type IV pili (TFP) contribute to swarming motility may depend on the bacterial species, strain, and culture conditions used in the assay.

Biofilm Assay

The observation results showed the presence of a purple ring-shaped formation on the microtiter plate wall (Figure 4). A purple ring formation on the microtiter plate wall is was observed after the incubated bacterial suspension is stained with crystal violet and rinsed with distilled water. This indicates indicated that *R. syzygii* subsp. *celebesensis* can form biofilm structures. Biofilm is a complex structure consisting of various extracellular polymeric substances, such as exopolysaccharides, proteins, and extracellular DNA. The formation of biofilm plays a crucial role in bacterial survival as it contributes to the virulence of pathogenic bacteria, enhances bacterial resistance to antimicrobial agents, and promotes colonization in specific habitats (Mori *et al.*, 2016). According to Conrad *et al.* (2011), bacterial ability to move near surfaces influences biofilm formation and development. Bacterial motility structures such as flagella and multiple type IV pili (TFP) can facilitate biofilm formation.

The results of this study are consistent with the research conducted by O'Toole and Kolter (1998b).

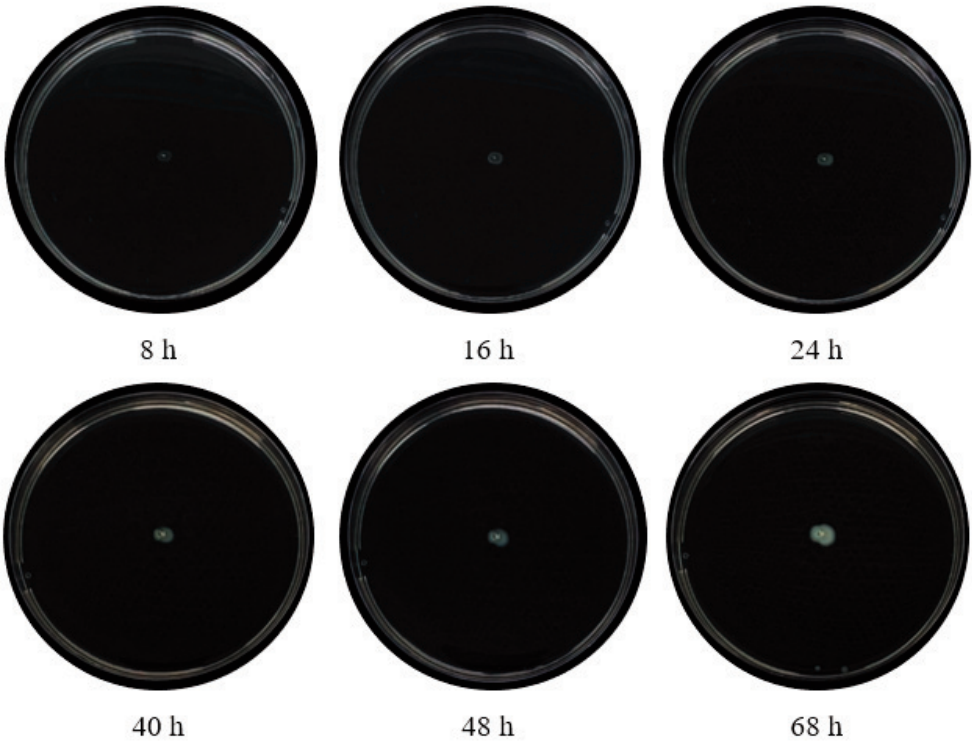

Figure 3. Swarming motility test results of *Ralstonia syzygii* subsp. *celebesensis* after 68 hours of observation, showing no significant development of the bacterial colony diameter on the Petri dish.

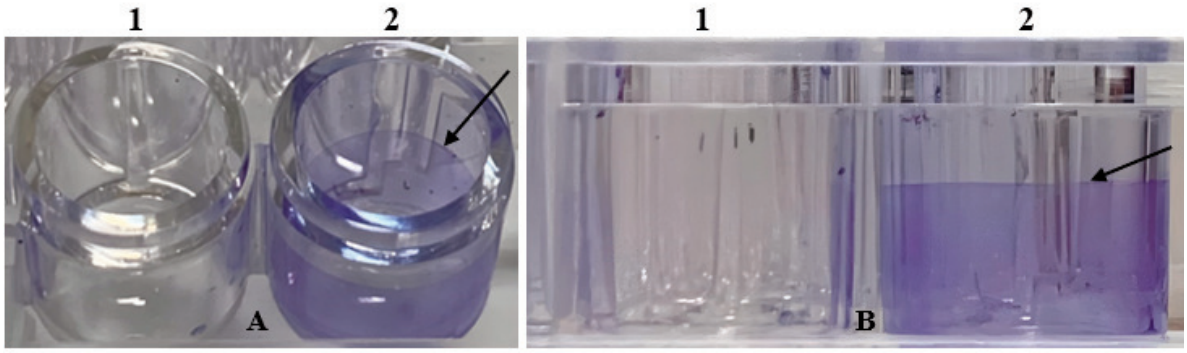

Figure 4. Biofilm formation testing using Casamino acid-Peptone-Glucose (CPG) agar medium as a control (1) and a suspension of *Ralstonia syzygii* subsp. *celebesensis* (2). The purple ring serves as an indicator of the biofilm formed by *R. syzygii* subsp. *celebesensis* on the microtiter plate, indicated by the black arrow; (A) top view, (B) side view.

Biofilm testing in both studies also showed that biofilm structures would form and be visible on the walls of microtiter plates made of PVC in a ring-like shape. This ring forms at the interface between air and the medium used when the suspension is incubated on the microtiter plate. Based on this, it can be concluded that bacteria are capable of forming biofilm on abiotic surfaces.

Biofilm formation is particularly important for bacteria that infect xylem tissue in plants (xylem-limited bacteria), including *R. syzygii* subsp. *celebesensis*. The bacteria produce an aggregate-like biofilm layer on the xylem vessels of plants, which can inhibit the flow of nutrients and water distributed throughout the plant's tissues. In *R. solanacearum*, a high bacterial population causes wilting symptoms due to the reduced flow of sap caused by a large number of bacterial cells and the exopolysaccharide (EPS) slime produced by the bacteria in the xylem vessels (Genin & Denny, 2012). This is similar to

the colonization of *R. syzygii* subsp. *celebesensis*, which causes banana plants to wilt. The bacterial colonization can be confirmed by the characteristic symptoms of banana blood disease, such as the reddish coloration inside the fruit flesh and the reddish-brown bacterial mass when the plant tissue is cut (Ho *et al.*, 2023).

Biofilms play a crucial role throughout the entire infection process of plant-pathogenic bacteria. In the initial stages, pathogens typically enter plants through natural openings like stomata, hydathodes, lateral root emergence sites, or wounds. However, in certain cases such as *R. syzygii* subsp. *celebesensis*, insect vectors are responsible for transmission. Once inside the plant, bacteria localize in the apoplast or move into the xylem. Imaging studies using bioluminescent or fluorescent markers in *R. solanacearum* and *X. campestris* pv. *campestris* have revealed bacterial migration into xylem vessels, which is accompanied by the activation of virulence genes (Monteiro *et al.*, 2012; Akimoto-Tomiyama *et al.*, 2014).

For vascular pathogens, the xylem offers a favorable environment for proliferation. As bacterial populations grow by utilizing xylem sap nutrients, initial biofilms begin to form. Continued growth and cell division result in the development of dense biofilm structures that, in later stages, can obstruct sap flow and disrupt the plant's normal physiological functions (Caldwell *et al.*, 2017; Khokhani *et al.*, 2017; Mina *et al.*, 2019).

Biofilm formation can differ between motile and non-motile bacteria. Bacterial motility structures, such as flagella, can play a significant role in adhesion by causing a more dynamic response in motile bacteria to surface properties compared to non-motile bacteria. Once bacteria adhere to a surface, motile bacteria can form biofilm more rapidly than non-motile bacteria. This may be due to the ability of motile bacteria to attract free bacteria through chemotaxis and quorum-sensing mechanisms (Gutman *et al.*, 2013). Quorum sensing is a communication process between bacterial cells that regulates cell density to initiate a response according to specific niche conditions (Fuqua *et al.*, 1994). Quorum sensing can also be defined as the process in which bacteria synthesize small molecules known as autoinducers, which are released passively or actively into the extracellular space. These molecules accumulate outside the cells, and their concentration is detected by bacteria through specific receptors (Moreno-Gámez *et al.*, 2023). Once a threshold concentration is reached, autoinducers trigger a signal transduction cascade that has been well-described in many bacterial species, regulating processes such as biofilm formation, virulence, competence, and sporulation (Miller & Bassler, 2001).

Bacteria do not always form biofilm layers. According to Moore-Ott *et al.* (2022), there are two conditions that cause autoinducer concentrations to remain below the threshold required for biofilm formation. First, nutrients available in the environment are consumed at very high rates, limiting the production of autoinducers by bacteria. Second, there is an increase in bacterial diffusion and dispersion due to environmental factors that affect the accumulation of autoinducers at a specific location.

Pellicle Assay

The observation results showed that *R. syzygii* subsp. *celebesensis* is capable of forming a pellicle layer on the surface of a liquid CPG medium (Figure 5). The pellicle layer began to form on the 8th day of observation. The pellicle formed as a thin, yellowish aggregate floating on the surface of the CPG medium in the test tube.

The pellicle is an ecological condition that benefits aerobic bacteria such as *R. syzygii* subsp. *celebesensis*. This occurs because the pellicle on the surface of the liquid medium provides access to high concentrations of oxygen from the air, in addition to the nutrients present in the liquid medium (Armitano *et al.*, 2014). The movement of *R. syzygii* subsp. *celebesensis* to the surface of the liquid medium can be attributed to the bacteria's attraction toward the air source, a phenomenon known as aerotaxis. According to Yamamoto *et al.* (2010), oxygen is one of the key factors for bacterial growth and survival at the liquid medium's air-liquid interface (ALI).

The pellicle formation observed in this study is was consistent with the research by Joko *et al.* (2007b), which investigated pellicle formation by *Dickeya dadantii*. In that study, the pellicle appeared as a thick white aggregate covering the surface of the SOBG medium in a test tube. Based on this article, it is understood that the pellicle plays a role in pro-

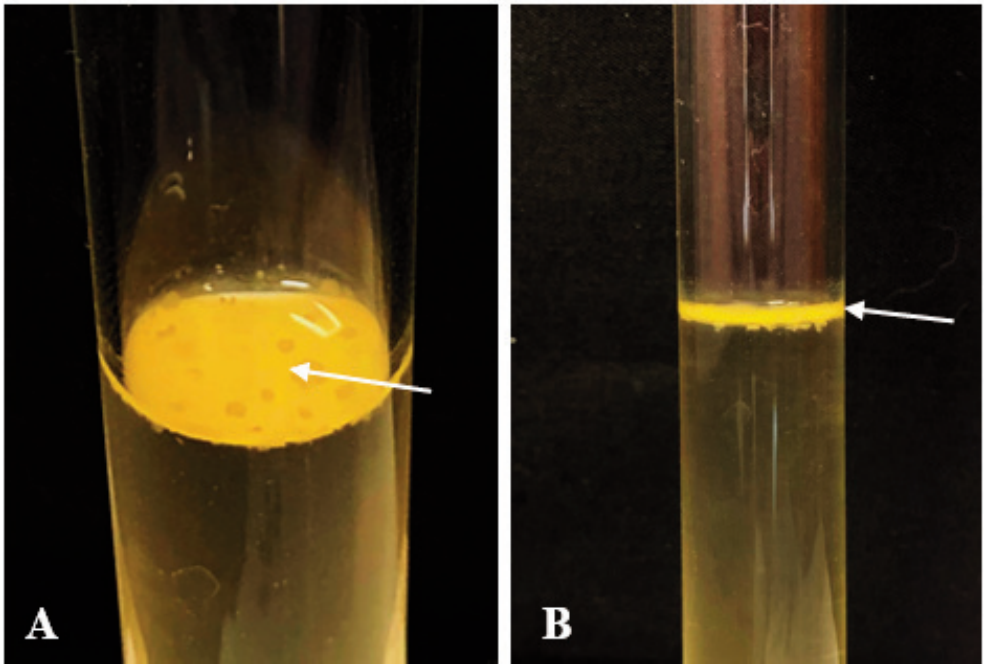

Figure 5. Pellicle layer formed by *Ralstonia syzygii* subsp. *celebesensis* on the surface of liquid media after 8 days, indicated by the white arrow; (A) top view, (B) side view.

tecting bacteria from competitors and providing resistance to antimicrobial agents in natural environments.

According to Monds and O'Toole (2009), the stages of biofilm and pellicle formation consist of planktonic growth, surface attachment, microcolony formation, biofilm development, and dispersal. Microcolony formation can occur through clonal growth of attached cells or active translocation of cells across surfaces, which then grow and merge to form biofilm structures. The pellicle structure formed on the surface of the liquid medium can dissolve over time, releasing cells from the biofilm (Mori *et al.*, 2016), which occurred in this study. The pellicle structure began to dissolve on the 12th day of observation. According to Morris & Monier (2003), biofilm is a collection of microorganisms embedded in an extracellular polymeric matrix that adhere to each other on a surface, enabling adaptation to fluctuating environmental conditions in a social manner. Based on the study by Hölscher *et al.* (2015), it can be inferred that in addition to oxygen availability, motility is another factor contributing to pellicle formation. The study revealed that pellicle formation was delayed in motility-deficient mutant strains. Despite the delay, the mutant strains exhibited a morphology similar to that of the wild-type, indicating that motility is not essential for the development of a robust pellicle. In non-motile strains, the delay in pellicle formation is likely due to the absence of directed movement toward the air-liquid interface. The increase in cell number may primarily result from the division of a few cells that reached the interface via passive, random Brownian motion, thereby slowing down the pellicle formation process. This is further supported by the finding that at high initial cell densities, the delay observed in *B. subtilis* Δ*hag* and *P. aeruginosa* Δ*flgK* mutants was no longer evident.

CONCLUSION

Based on the research conducted, it can be concluded that *R. syzygii* subsp. *celebesensis* is suspected to perform passive motility in the form of sliding motility. However, it is unable to move via swarming motility and possesses the ability to form biofilm structures and a pellicle layer.

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