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Isolation of Endophytic Bacteria from Melon Root and Evaluation of Their Antagonistic Activity Against *Acidovorax citrulli*

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ABSTRACT

Endophytic bacteria are a potential biocontrol agent to control plant diseases. Controlling plant disease using bactericides has negative impacts, including the death of other organisms on plants. This research aimed to identify potential endophytic bacteria from melon roots for biocontrol against *Acidovorax citrulli*, the pathogen causing Bacterial Fruit Blotch (BLB) disease in the Cucurbitaceae family. A total of 11 endophytic bacteria were isolated from healthy melon roots. The isolates exhibited similar colony morphology with white, circular, convex elevation. Physiology and biochemistry tests revealed 8 isolates as Gram-negative and catalase-positive, while the remaining 3 were identified as Gram-positive and catalase-negative. A fluorescence test on King's B media indicated that the Gram-negative isolates could belong to the *Pseudomonas* genus. This study also confirmed *Acidovorax* sp. cultures isolated from infected melon plants many years ago as *A. citrulli*. Assessment of pathogenicity in the melon plants showed that *A. citrulli* isolate N2 was the most pathogenic. Based on the *in vitro* inhibition test, all the Gram-negative isolates formed inhibition zones ranging from 1.94-4.41 mm, suggesting their potential to inhibit the growth of *A. citrulli*. The EB_6 isolate exhibited the highest inhibition zone at 4.41 mm ±0.28. five of the eight isolates tested (EA_1, EB_1, EB_3, EB_4, and EB_6) did not exhibit any Hypersensitive Response (HR) reaction in the tobacco leaves, thus can potentially be used as a biocontrol agent against BLB disease in melon plants. Further studies are required to determine the species identity of the isolates and explore their application as biocontrol agents.

1. Introduction

Melon (*Cucumis melo* L.) is an annual herbaceous plant of the Cucurbitaceae family that can be grown in high and lowlands (Endl *et al.* 2018). In Indonesia, melon is an essential commodity. However, the production of melon decreased by 14.09% from 138.177 tons in 2020 to 118.696 tons in 2022 (BPS 2024). The pathogens that can attack melons include *Acidovorax citrulli, Puccinia xanthii*, and *Fusarium* sp. (Kumagai *et al.* 2014; Pineda *et al.* 2018; Ishak and Daryono 2020). Bacterial Fruit Blotch (BFB), caused by *Acidovorax citrulli*, poses a significant threat to cucurbits production, especially melon and watermelon (Horuz 2021) due to its diverse range of symptoms and its substantial impact on yield. This broad host range enhances the potential for widespread damage to crops. *A. citrulli* is a member of the β-proteobacteria class and the *Acidovorax* genus. It is a Gram-negative, aerobic, mesophilic, soil-borne bacterium (Husni *et al.* 2021). Classified as a quarantine microorganism category A2, A. citrulli has spread in Indonesia (IQA 2008), and primarily detected in the Magelang and Yogyakarta areas (Windari *et al.* 2015).

The manifestation of BFB includes various symptoms such as brown to reddish wet spots on leaves, rotting and cracking of melon fruit, and lesions on cotyledons (Horuz *et al.* 2014; Windari *et al.* 2015). These symptoms serve as visible indicators of the disease's presence and progression. The damaging effects of BFB on melon production are significant, with potential yield reductions ranging

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from 40 to 100% (Alves *et al.* 2018). *A. citrulli* can persist in soil and spread through various means, including contaminated seeds, soil, irrigation water, and emerging seedlings (Latin and Hopskin 1995; Bahar *et al.* 2009). These multiple, complicated transmission paths pose a significant challenge to effectively managing and controlling the disease's spread. The management of BFB disease typically involves using bactericides, which can effectively control the pathogen's spread. However, dependence only on bactericides may lead to various adverse effects. These include harm to beneficial organisms, environmental pollution, and pathogen resistance (Burdon *et al.* 2016).

Effective strategies are crucial to controlling the spread of BFB disease and protecting melon crops. Currently, using biological agents for disease control, particularly endophytic bacteria, is a promising approach that has yielded encouraging results across various crops. Several studies have explored the effectiveness of different endophytic bacteria in combating plant pathogens. Many studies have shown that certain endophytic species, such as *Curtobacterium flaccumfaciens, Microbacterium oxydans, Pseudomonas oryzihabitans, Pseudomonas fluorescens, Pseudomonas chlororaphis* YL-1 can inhibit the growth of *A. citrulli* (Horuz and Aysan 2017; Jayanti and Joko 2020: Liu *et al.* 2021; Horuz 2021). In another study, Gu *et al.* (2022) reported the potential of Pseudomonas protegens Pf-5 in inhibiting the growth and pathogenicity of brown rot on maize leaves, while Akbaba and Ozaktan (2018) reported on the potential of *Ochrobactrum pseudintermedium* in suppressing angular leaf spot disease in cucumber plants. This study aimed to identify endophytic bacteria from melon roots that can potentially suppress *A. citrulli*. The potential of biological control agents for managing BFB is promising, offering a sustainable and ecologically sound alternative to traditional chemical methods.

2. Materials and Methods

The research was conducted at the Bacteriology and Molecular Biology Laboratory, Central Agricultural Quarantine Standard Testing Center (Balai Besar Uji Standar Karantina Pertanian (BBUSKP)). The melon (*Cucumis melo* L. variety 'Sky Rocket') was obtained from the Kamal Muara Nursery, North Jakarta. Endophyte bacteria were isolated from the melon roots of a six-week-old plant (Code A) and a five-week-old plant (Code B).

This research is structured into three main steps to comprehensively study Bacterial Fruit Blotch (BFB) disease and potential biocontrol agents: (1) Isolation and identify endophytic bacteria using morphological, physiological and biochemical characterization; (2) Verification of *A. citrulli* that was obtained from infected melon plants in the Kulon Progo area, Yogyakarta in January 2017 using specific primers; (3) Pathogenicity test in melon plant; (4) *In vitro* inhibition testing of endophytic bacteria against *A. citrulli.*

2.1. Isolation and Identify Endophytic Bacteria from Melon Roots

The isolation method of endophytic bacteria is based on the protocols outlined by El-Deeb *et al.* (2012) and Horuz and Aysan (2017). Melon root samples are collected and cleaned thoroughly using running water to remove any external debris or soil particles. The samples undergo a sequential sterilization process with alcohol (70%) for 3 minutes, followed by 2% NaOCl for 3 minutes, then rinsed with sterile water three times. Control samples are prepared using the same method, with the final rinse water used to spread onto sterile plates. All samples were plated on Tryptic Soy Agar (TSA) (Merck).

Melon root samples are carefully cut into small pieces (1 gram) and placed in a thick plastic bag. Phosphate Buffer Saline (PBS) is added to the samples (10 ml) and crushed using a pestle. The extract is then shaken at 150 rpm for 30 minutes to ensure a comprehensive release of endophytic bacteria from the plant tissue. The extract is then diluted at various dilutions (10^{-1}, 10^{-3}, 10^{-5}, 10^{-7}) using sterile water to obtain different bacterial concentrations. Aliquots (0.1 ml) of each dilution are plated onto TSA media in triplicate. The plates were incubated for five days at 28°C to allow bacterial colonies to grow. Morphological characterization involves observing colony characteristics such as colour, shape, margin, and elevation. Physiological and biochemical characterization includes conducting Gram staining, catalase tests, oxidative/fermentative (OF) tests (Schaad *et al.* 2001) and fluorescence tests at 366 nm wavelength to identify pigmented and non-pigmented bacteria (Yoon *et al.* 2021).

2.2. Molecular Verification Of *Acidovorax* Isolates

Six *Acidovorax* isolates (N_1, N_2, $O_{1.1}$, $O_{1.2}$, $O_{2.1}$ and $O_{2.2}$) were obtained from the BBUSKP collection and grown in King's B media at 28°C for 48 hours. The DNA extraction was carried out based on Gomes *et al.* (2000) and Kejani *et al.* (2009). After incubation, 1.5 ml of bacterial culture was transferred into a centrifuge tube. The tube was centrifuged for 2 minutes until a pellet of bacterial cells formed at the bottom. The supernatant is carefully discarded. The pellet was added 600 µL of Cetyltrimethylammonium (CTAB) solution, 0.2% β-mercaptoethanol, and 50 mg of Polyvinylpyrrolidone (PVP-40). The tube was then incubated at 55°C for 25 minutes to facilitate cell lysis and genomic DNA release. Following cell lysis, 450 µL of chloroform: isoamyl alcohol (24:1) was added to the tube. The tube was centrifuged at 15,000 g for 3 minutes to separate the mixture into layers. The upper aqueous phase, containing the DNA, was transferred to a new tube, leaving behind the organic phase and debris. DNA precipitation was carried out by adding one volume of cold isopropanol to the transferred aqueous phase. The tube was then incubated for 15 minutes at -20°C and centrifuged at 15,000 g for 5 minutes. The supernatant was discarded and the DNA pellet was washed with 500 µL of 70% ethanol to remove residual contaminants. The tube is centrifuged at 15,000 g for 5 minutes to pellet the DNA, and the ethanol is carefully removed. The DNA pellet is allowed to air-dry briefly before being resuspended in 50 µL of nuclease-free water.

The PCR amplification was carried out based on the method by Walcott *et al.* (2003) using species-specific primers, SEQID4m (5'-GTC-ATTACTGAATTTCAACA-3') and SEQID5 (5'-CCCTCCACCA-ACCAATACGCT-3'), targeting the 16S-23S ITS DNA ribosomal region of *A. citrulli* with an expected size of 246 bp. The PCR reaction mixture is prepared in a 25 µL volume, consisting of 1 µL of DNA template, 12.5 µL of Master Mix, 1 µL of each primer at a concentration of 10 µM each, and 9.5 µL of nuclease-free water. The PCR machine was programmed as follows: 95°C (5 minutes), 35 cycles of 95°C (30 seconds), 53°C (30 seconds), and 72°C (30 seconds) and final elongation step at 72°C (5 minutes). The PCR products were electrophoresed on 1% agarose gel at 70 volts for 1 hour. The agarose gel was stained with ethidium bromide (0.5 g/ml) solution and visualized under UV light.

2.3. Patogenity Testing of *A. citrulli* in Melon Plants

All six isolates of *A. citrulli* were tested for pathogenicity and ability to infect melon plants by inducing BFB disease symptoms. The method used was based on Horuz *et al.* (2014) with modifications. The isolates were cultured in King's B medium for 48 hours at 28°C. There were two infected plants for each treatment. One leaf of a three-week-old of each melon plant was injured by making three punctures using a syringe along the central vein, then sprayed with a bacterial suspension of *A. citrulli* with an Optical Density (OD) of 0.2 at A_{6000}. The leaves were then wrapped in clear plastic to maintain high humidity levels and incubated for 2-7 days. Control plants underwent a similar process of leaf injury but were sprayed with sterile water. The pathogenicity test was positive if the plants exhibited BSB symptoms consistent with necrotic leaf spots, which may have chlorotic halos (Hopkins *et al.* 2000). Symptomatic leaves were sampled and subjected to DNA extraction using the protocol described previously. The isolates that induced the most extensive symptoms were selected for further study.

2.4. *In-Vitro* Antagonistic Assay of Endophytic Bacteria Against *A. citrulli*

The endophytic bacteria are streaked onto Petri dishes containing *A. citrulli* inoculum in three repetitions for each treatment. The Petri dishes are then incubated at 28°C for 48 hours for optimal growth and interaction between the endophytic bacteria and *A. citrulli.* Any bacterial isolates capable of inhibiting *A. citrulli* will show an inhibition zone, which appears as clear areas devoid of bacterial growth around the colonies of the endophytic bacteria. The screening test will be continued by measuring the inhibition zone formed from the colony's edge to the clear zone's outer edge to ensure the accuracy of the results.

2.5. In Planta Hypersensitivity Assay

To determine whether an isolate is capable of inducing a hypersensitive reaction in plants, tobacco plants of the Darmawangi variety, aged 14 weeks post-transplanting from Tanjungsari, Sumedang, West Java, were used. The tobacco plant has been widely used to study plant-pathogen virulence, including bacteria (Selangga and Listihani 2021).

The small genome size of tobacco plants used for hypersensitivity tests provides clear, practical, and efficient advantages (Edwards *et al.* 2017). The bacterial suspensions were infected onto the tobacco leaves and observed for hypersensitive reactions, such as chlorosis or necrosis. The bacterial suspension (A_{600}: 0.2 OD) is infiltrated into tobacco leaves (*Nicotiana tabacum* L.) with a syringe. The negative control test is carried out by infiltrating sterile distilled water, and the positive control test is by infiltrating the suspension of the pathogen *Acidovorax citrulli*. The infiltrated leaves are then incubated for 48 hours. The presence of chlorosis or necrosis in the infiltrated area after three weeks of infection indicates the pathogenic nature of the bacteria.

3. Results

3.1. Isolation and Identification of Endophytic Bacteria from Melon Roots

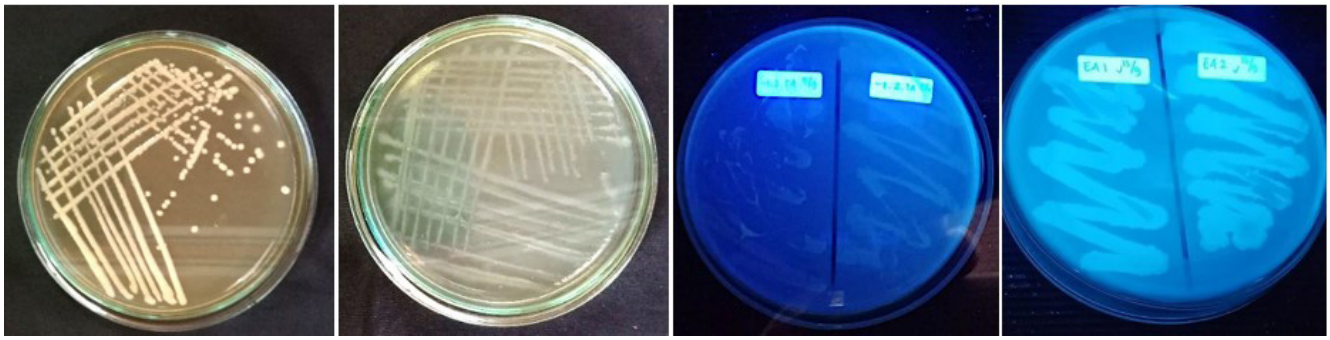
Results of the isolation and characterization of endophytic bacteria were obtained from 11 isolates (EA_1, EA_2, EA_3, EA_4, EA_5, EB_1, EB_1, EB_2, EB_3, EB_4, EB_5, EB_6). Based on the morphological characteristics observed, most bacterial isolates from melon roots displayed white colonies (8 isolates) and white to yellow (3 isolates) and exhibited circular colony shapes. All isolates exhibited convex elevations. Based on physiological and biochemical analysis, 8 (eight) isolates are gram-negative; all eight isolates are obligate aerobes (+/-), and 3 (three) isolates are facultative aerobes (+/+). All isolates showed catalase activity, and 8 (eight) isolates exhibited fluorescence (Table 1; Figure 1).

3.2. Validity Testing of *Acidovorax* Isolates Using Molecular Markers

This study used six isolates of *Acidovorax* from the BBUSKP Collection, which had been stored for six years in sterile water at room temperature. The PCR amplification for all six isolates resulted in a DNA band at the expected size of 246 base pairs (bp) (Figure 2), which confirms the identity of the six isolates as *Acidovorax citrulli*.

Table 1. Morphological, physiological and biochemical characteristics of endophytic bacteria isolated from melon roots

Isolate code	Morphological characteristic					Physiological and biochemical characteristic			
	Age of plant	Colony color	Colony shape	Colony elevation	Colony margin	Gram	OF	Catalase	Fluoro-scence
EA_1	Six weeks (A)	White	Irregular	Convex	Filiform	−	+/−	+	+
EA_2	Six weeks (A)	White to yellow	Circular	Convex	Entire	−	+/−	+	+
EA_3	Six weeks (A)	White	Irregular	Convex	Undu-late	−	+/−	+	+
EA_4	Six weeks (A)	White to yellow	Circular	Convex	Entire	+	+/+	+	−
EA_5	Six weeks (A)	White	Circular	Convex	Undu-late	+	+/+	+	−
EB_1	Five weeks (B)	White	Irregular	Convex	Undu-late	−	+/−	+	+
EB_2	Five weeks (B)	White to yellow	Circular	Convex	Undu-late	−	+/−	+	+
EB_3	Five weeks (B)	White	Circular	Convex	Filiform	−	+/−	+	+
EB_4	Five weeks (B)	White	Circular	Convex	Entire	−	+/−	+	+
EB_5	Five weeks (B)	White	Circular	Convex	Entire	+	+/+	+	−
EB_6	Five weeks (B)	White	Circular	Convex	Serrate	−	+/−	+	+



A B C D

Figure 1. (A) Representative figure of morphological characteristic of bacterial isolates EA_4, (B) EB_6, (C) biochemical characteristic of bacterial isolate (EA_4, EA_5) that did not produce fluorescent pigment, and (D) bacteria isolate (EA_1, EA_2) that produce fluorescent pigment

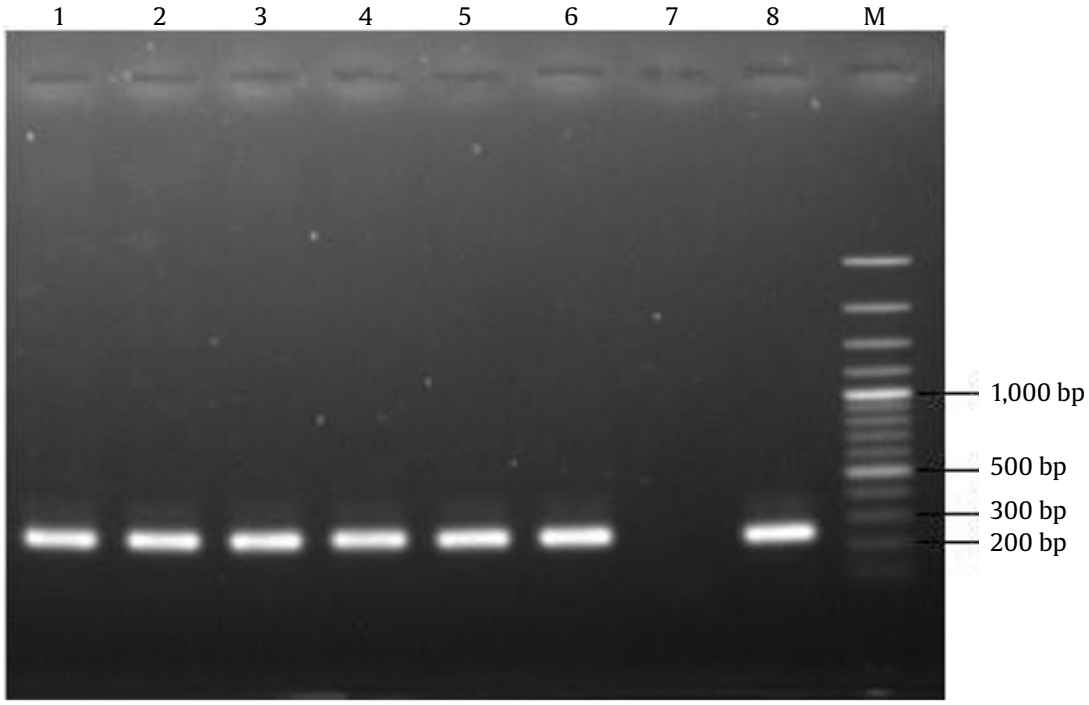


Figure 2. Visualization of electrophoresis from *A. citrulli* isolates (1) N_1, (2) N_2, (3) $O_{1.1}$, (4) $O_{1.2}$, (5) $O_{2.1}$, (6) $O_{2.2}$, (7) negative control (sterile distile water), (8) positive control (confirmed *A. citrulli*), (M) DNA ladder

3.3. Pathogenicity Testing of *Acidovorax citrulli* in Melon Plants

The pathogenicity test conducted on melon plants showed that the control plants (uninfected) did not exhibit any symptoms after seven days of infection. However, all six *A. citrulli* isolates induced visible disease symptoms upon infection (Figure 3), thus confirming their ability to infect them. The first symptom is chlorosis around the inoculation site (the leaf's central vein), which spreads quickly and causes the surrounding tissue to become necrotic. Moreover, the pathogens also attack melon cotyledons, forming wet spots that eventually lead to chlorosis and necrosis. Among the six *A. citrulli* isolates, N_2 showed the most severe symptoms. The cotyledons and leaves of melon plants infected with N_2 isolate exhibited necrosis and wilting until the plant died (Figure 3C). Hence, the N_2 isolate was chosen for subsequent stages of the following experiment. The PCR amplification using *A. citrulli*-specific primers confirmed the presence of the individual isolates in the inoculated plant samples (Figure 4).

3.4. Antagonistic of Endophytic Bacteria to *A. citrulli* and Hypersensitive Response Testing

This experiment focused on identifying endophytic bacterial isolates from melon plants that can inhibit the growth of *Acidovorax citrulli* N_2, indicated by an inhibition zone around the bacterial colonies. Through *in vitro* inhibition screening, it was observed that 8 (EA_1, EA_2, EA_3, EB_1, EB_2, EB_3, EB_4, and EB_6) formed an inhibition zone, while the remaining three isolates (EA_4, EA_5, EB_5) did not. Isolate EB_6 showed the largest inhibitory zone (4.41±0.28 mm), and isolate EB_3 exhibited the smallest inhibitory zone (1.94±0.18 mm) (Table 2). Based on the physiological and biochemical characteristics in Table 1, the eight isolates forming the inhibition zones are likely from the genus *Pseudomonas*.

The results of the hypersensitive test conducted on tobacco plants showed that three endophytic bacterial isolates (EA_2, EA_3, EB_2) exhibited symptoms of chlorosis (Table 2; Figure 5). This observation indicates that these isolates induced a positive reaction, making them pathogenic on plants and therefore excluded from further testing.

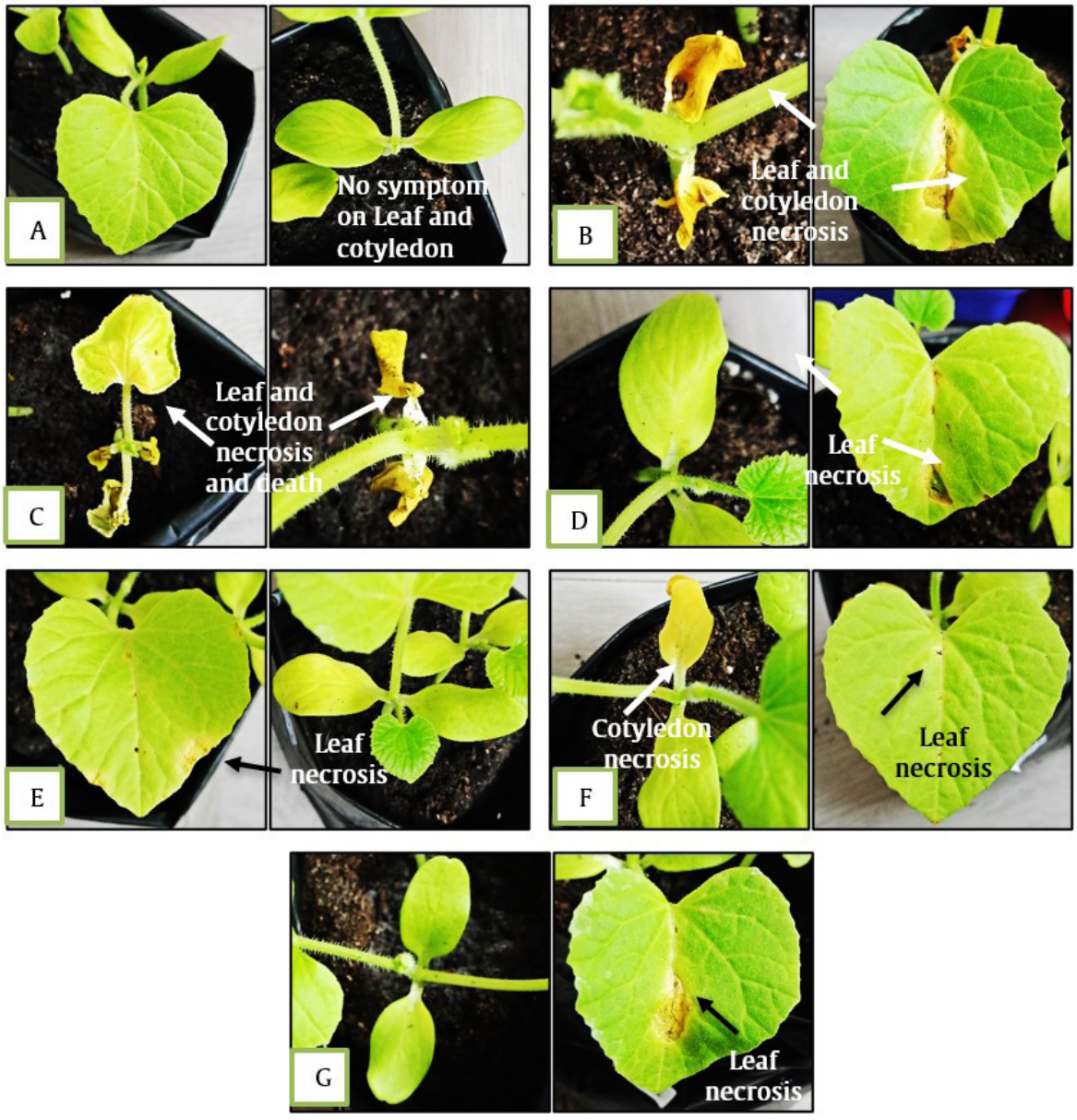


Figure 3. Pathogenity of *A. citrulli* on melon leaves (A) negative control, (B) *A. citrulii* N_1, (C) *A. citrulli* N_2, (D) *A. citrulli* $O_{1.1}$, (E) *A. citrulli* $O_{1.2}$, (F) *A. citrulli* $O_{2.1}$, (G) *A. citrulli* $O_{2.2}$

4. Discussion

The rich plant biodiversity in Indonesia presents a wealth of opportunities to discover endophytic bacteria. Once isolated and identified, these microorganisms could potentially be utilised to control plant diseases. In this study, endophytic bacteria were isolated on the roots of melon plants because root organs have the largest population of endophytic bacteria (Ryan *et al.* 2008; Vandana *et al.* 2021). The roots release amino acids and sugars into the soil, which attracts microorganisms to colonise the roots (Afzal *et al.* 2019; Firdous *et al.* 2019).

The 11 endophyte bacteria isolated in this study exhibited predominantly white, circular colony shape and convex elevation (Table 1). These morphological characteristics can be influenced by various factors such as gene expression, media composition, pH, temperature, and oxygen availability (Jeanson *et al.* 2015). The biochemical characteristics of the bacteria (Table 1), provide a comprehensive understanding of their diversity

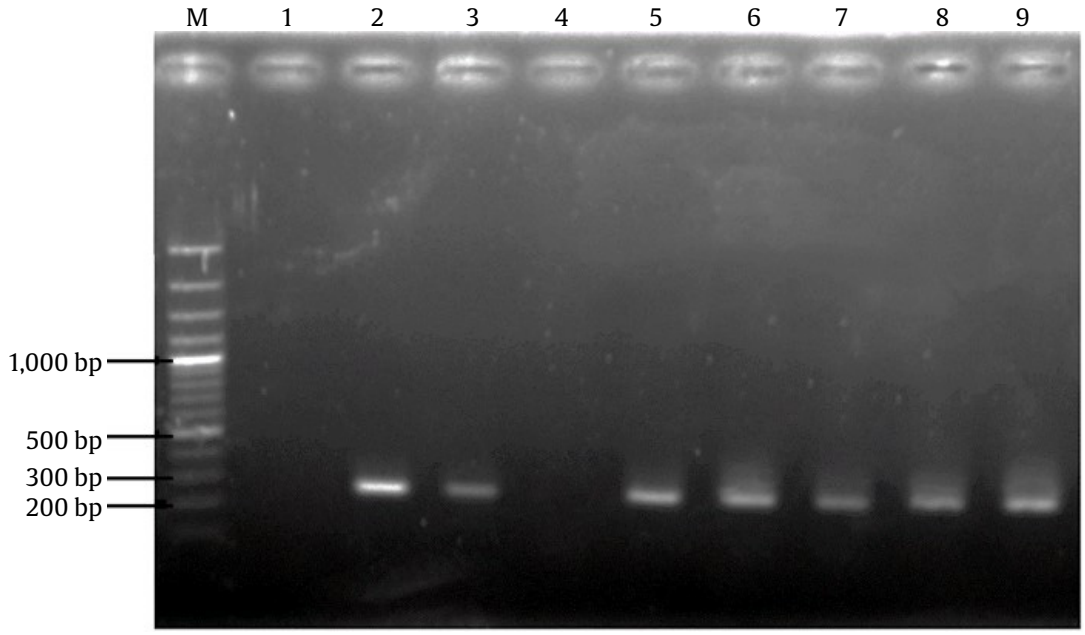


Figure 4. Confirmation of *A. citrulli* isolates in the melon leaves showing BFB symptoms by PCR. (M) DNA ladder, (1) negative control, (2) positive control, (3) *A. citrulli* N_1, (4) sterile distile water (negative control), (5) *A. citrulli* N_2, (6) *A. citrulli* $O_{1.1}$, (7) *A. citrulli* $O_{1.2}$, (8) *A. citrulli* $O_{2.1}$, and (9) *A. citrulli* $O_{2.2}$

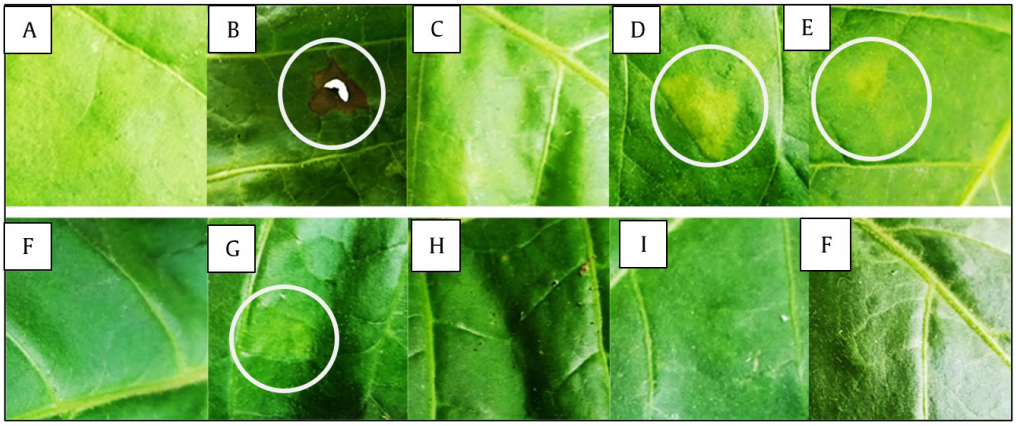


Figure 5. Tobacco leaves with necrosis symptom showed a hypersensitive reaction after inoculation with (A) Aquadest (HR negative), (B) *Acidovorax citrulli* N_2 (HR-positive), (C) EA_1 (HR negative) (D) EA_2 (HR-positive), (E) EA_3 (HR-positive (F), EB_1 HR negative, (G) EB_2 HR-positive, (H) EB_3 HR negative, (I) EB_4 HR negative, (J) EB_6 HR negative

Table 2. The inhibition effect of endophytic bacteria isolates against pathogen *A. citrulli* N_2 and hypersensitive respone (HR) test in tobacco leaves

Isolate code of endophytic bacteria	Inhibition zone (mm) ± SE	Hypersensitive Response (HR)
EA_1	2.43 ± 0.14^{cd}	-
EA_2	2.90 ± 0.15^{bc}	+
EA_3	3.00 ± 0.17^{b}	+
EB_1	2.23 ± 0.16^{d}	-
EB_2	2.85 ± 0.14^{bc}	+
EB_3	1.94 ± 0.18^{d}	-
EB_4	2.95 ± 0.13^{bc}	-
EB_6	4.41 ± 0.28^{a}	-

Numbers followed by different letters indicate significant differences based on Duncan's Multiple Range Test (DMRT) at the 5% level

and traits, which can be used to predict their potential identity. Gram-negative bacteria are known for their diverse metabolic capabilities and pathogenic potential, while obligate aerobes require oxygen for growth (Prescott *et al.* 1996). Catalase activity indicates the ability to break down hydrogen peroxide, which is common in aerobic organisms. The fluorescence observed suggests the presence of pigments like pyoverdine, produced by *Pseudomonas* spp. King's B media contain protease and peptone, which act as an energy source and promote pyoverdine pigment production (Ahmad *et al.* 2008). Fluorescent *Pseudomonas* spp. have a

competitive ability to defeat other microorganisms for nutrients and space in various plant-associated environments, diverse metabolic capabilities and pathogenic potential (Meyer 2000; Cézard *et al.* 2015).

Validating the identity of isolates is crucial to ensure the accuracy and reliability of research findings, especially in cases where contamination or misidentification may occur during collection, long-term storage, or handling. PCR assays with specific primers offer a rapid and accurate means of identifying bacterial species and do not require a long time to detect the presence of target bacteria (Cho *et al.* 2015). In this study, the validation of *Acidovorax* isolates using specific primers demonstrates a targeted approach for confirmation. The choice of primers, with high specificity for detecting *A. citrulli* in Cucurbitaceae plants, enhances the reliability of the validation process (Walcott *et al.* 2003). The primers are based on the 16S-23S ITS region of the ribosomal DNA of *A. citrulli* where one or more tRNA genes play an essential role in the transcription process (Cho *et al.* 2015).

The observation that the *A. citrulli* isolate N_2 exhibited the most severe symptom with necrosis and wilting in cotyledon and leaves, compared to other isolates (Figure 3C), suggests differences in pathogenicity among isolates. Leaf lesions are light brown to reddish-brown and often spread along the tobacco leaf's midrib (Figure 3B, D, and G). Differences in the severity of disease symptoms could be caused by gene expression variations, which play a role in pathogenicity and virulence (Satoh *et al.* 2011). Gram-negative pathogenic bacteria often utilize the type III secretion system (T3SS) as a pathogenicity factor. This system facilitates the injection of effector proteins directly into the host cell cytoplasm, contributing to bacterial virulence mechanisms (Ji and Dong 2015). The hrp gene, which regulates the T3SS, is particularly critical (Wang *et al.* 2018). Variations in this gene's presence or expression levels can influence the virulence of bacterial isolates. Eckshtain-Levi *et al.* (2014) suggest that different strains of *A. citrulli* possess distinct T3SS protein effectors, leading to variations in virulence levels among the strains. Pathogenicity testing is crucial to ensure that the characteristics of bacteria remain unchanged over time, especially during continuous subculture or long-term storage. Altered pathogenicity and virulence capabilities in bacteria could lead to more severe diseases, increased treatment resistance, or even new diseases (Domenech and Reed 2009; Duangurai *et al.* 2020). In this study, the presence of an expected band at 246 bp in agarose gel electrophoresis and disease symptoms confirmed the presence of *A. citrulli* (Figure 4) and their ability to infect melon plants.

In this study, the size of the inhibition zones varied across the different endophytic bacterial isolates, ranging from 1.94 to 4.41 mm (Table 2; Figure 6), reflecting the different levels of the effectiveness of the endophytic bacteria in suppressing the growth of *A. citrulli*. The endophyte bacteria isolate EB_6 showed the largest inhibitory zone size. However, the inhibition zone obtained in this study was smaller than previously reported. Several studies have reported the antagonistic activity of *Pseudomonas* spp. to inhibit the growth of *A. citrulli* by *in vitro* assay with inhibition zones ranging from 6 to 13 mm (Horuz and Aysan 2017). According to Sadrati *et al.* (2013), the inhibition zone size is directly proportional to the ability of bacteria to inhibit pathogens. This variation in inhibition

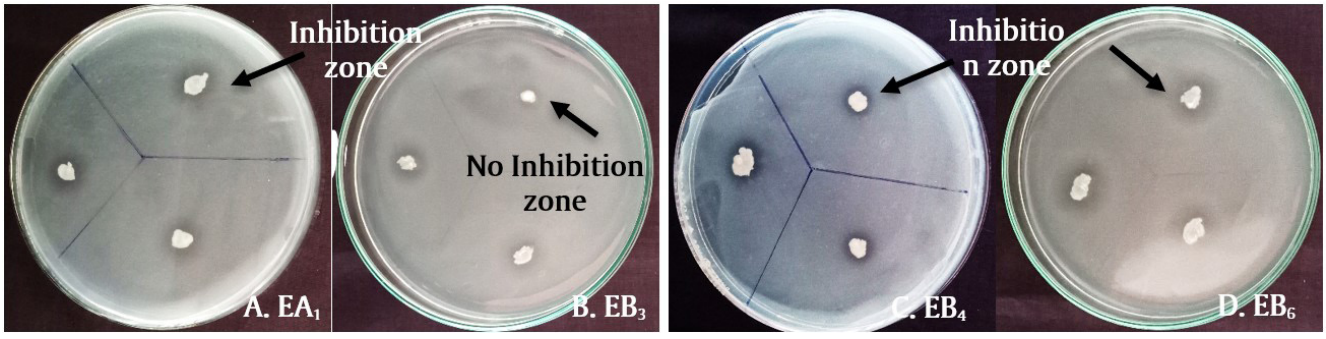


Figure 6. Representative figure of inhibition effect of endophyte bacteria isolates EB_3 (A), EB_4 (B) and EB_6 (C) against *A. citrulli* N_2

zones could be noted that several species of *Pseudomonas* produce different types of antibiotics. The formation of the inhibition zone is a direct result of *Pseudomonas* bacteria's ability to produce antimicrobial components, such as hydrogen cyanide (HCN), phenazine-1-carboxylic acid (PCA), 2,4-diacetyl phloroglucinol (2,4-DAPG), Pyoluteorin (Plt), pyrrolnitrin, rhizoxin, and hexyl -5-propyl resorcinol (HPR) (Loper *et al.* 2012; Pellicciaro *et al.* 2022; Gu *et al.* 2022).

Plants have a hypersensitive defence response, a mechanism that triggers rapid cell death at the site of pathogen invasion. This mechanism is associated with pathogen resistance and protects the plant from further harm (Slusarenko *et al.* 2000; Balint-Kurti 2019; Thakur *et al.* 2019). The hypersensitive response is an important test that separates plant pathogenic bacteria from saprophytes. Current standard protocols use tobacco as an indicator for Gram-negative and Gram-positive phytopathogenic bacteria, and infection is carried out by infiltrating bacterial suspensions into intact leaves (Umesha *et al.* 2008). According to Edwards *et al.* (2017), using tobacco plants for the hypersensitivity test, due to their small genome size, offers practical and efficient advantages. It may facilitate the screening of endophytic bacterial isolates for their potential to induce hypersensitive response (HR) reactions. If the tested endophytic bacterial isolates do not trigger HR reactions in tobacco plants, there may be optimism that these isolates will not induce similar reactions in plants with larger genomes.

The endophyte bacteria isolate EA_2, EA_3, and EB_2 induced an HR response (positive) in the tobacco plants by showing necrosis lesions in infected leaves (Figure 6), and they were categorized as pathogenic on plants. This study identified several endophytic bacteria isolates that likely belong to the genus Pseudomonas based on phenotypic characterization and fluorescence production. Isolates EA_1, EB_1, EB_3, EB_4, and EB_6 were able to inhibit *A. citrulli* growth *in vitro*, suggesting their potential in controlling Bacterial Fruit Blotch (BFB) disease. This finding is a promising strategy for using endophytic bacteria for disease control in agriculture. However, further study is required to identify the bacterial species and the antimicrobial components responsible for inhibiting *A. citrulli*. It is also essential to validate the efficacy of these isolates in greenhouse and field conditions.

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