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Antagonistic Potential of Rhizosphere Bacteria from Citrus Plants in East Kalimantan against *Lasiodiplodia theobromae*

(Potensi Antagonistik Bakteria Rhizosfera daripada Tumbuhan Limau di Kalimantan Timur terhadap *Lasiodiplodia theobromae*)

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ABSTRACT

East Kalimantan is one of the central areas for the development of Siamese oranges (*Citrus nobilis*) in eastern Indonesia. However, diplodia stem rot disease caused by *Lasiodiplodia theobromae* causes low productivity. Alternative control of those pathogens using indigenous antagonist bacteria is environmentally friendly. This study aims to analyse the diversity of rhizosphere bacteria in citrus plants in dry land and swamps from East Kalimantan and evaluate their potential to inhibit the growth of *L. theobromae in vitro*. The research consisted of rhizosphere bacteria isolation, analysis of bacterial diversity, potency assay of each isolate to inhibit the growth of *L. theobromae,* and identification of potential bacteria isolates based on 16S rDNA similarity. The results showed that 17 isolates of non-pathogenic rhizosphere bacteria, three of which, namely T4, T13, and T14, have the highest potency to inhibit the growth of *L. theobromae*. Among those isolates, the T13 bacterial isolate had the highest potency to inhibit that pathogenic fungus at logarithmic and stationary growth phases. Isolate T4, based on 16S rDNA sequence similarity, was identified as *Bacillus subtilis*, while T13 and T14 were identified as *Pseudomonas aeruginosa*.

Keywords: *Bacillus subtilis*; *Citrus nobilis; Lasiodiplodia theobromae; Pseudomonas aeruginosa*; rhizosphere bacteria

ABSTRAK

Kalimantan Timur merupakan salah satu kawasan pusat pengembangan limau Siam (*Citrus nobilis*) di wilayah Indonesia Timur. Walau bagaimanapun, penyakit reput batang diplodia yang disebabkan oleh *Lasiodiplodia theobromae* telah menyebabkan produktiviti yang rendah. Kawalan alternatif terhadap patogen tersebut menggunakan bakteria antagonis asli merupakan pendekatan yang mesra alam. Penyelidikan ini bertujuan untuk menganalisis kepelbagaian bakteria rhizosfera pada tumbuhan sitrus yang terdapat di tanah kering dan paya dari Kalimantan Timur dan menilai potensinya untuk menghalang pertumbuhan *L. theobromae* secara *in vitro*. Penyelidikan ini meliputi pemencilan bakteria rhizosfera, analisis kepelbagaian bakteria, ujian potensi setiap pencilan untuk menghalang pertumbuhan *L. theobromae* dan pengenalpastian pencilan bakteria berpotensi berdasarkan 16S rDNA. Hasil kajian menunjukkan 17 pencilan bakteria rhizosfera bukan patogen, tiga daripadanya iaitu T4, T13 dan T14 mempunyai potensi yang paling tinggi untuk menghalang pertumbuhan *L. theobromae*. Antara pencilan tersebut, bakteria T13 mempunyai potensi tertinggi untuk menghalang kulat patogen pada fasa pertumbuhan logaritma dan pegun. Pencilan T4, berdasarkan persamaan jujukan 16S rDNA dikenal pasti sebagai *Bacillus subtilis*, manakala T13 dan T14 dikenal pasti sebagai *Pseudomonas aeruginosa*.

Kata kunci: *Bacillus subtilis*; bakteria rhizosfera; *Citrus nobilis; Lasiodiplodia theobromae; Pseudomonas aeruginosa*

INTRODUCTION

Citrus is one of the most economically valuable fruit crops in Indonesia, with the Siam variety widely cultivated due to its adaptability across various altitudes, including in East Kalimantan. However, its production in the region has recently declined from 113.006 to 83.955 quintals (BPS 2023). One of the major threats is gummosis, a stem rot disease caused by *Lasiodiplodia theobromae*, a fungal pathogen known for its wide host range and distribution in tropical and subtropical areas (Salvatore, Andolfi & Nicoletti 2020). The disease causes wet lesions, bark splitting, and exudation of golden brown sap (Dwiastuti, Agustina & Triasih 2016) and currently affects 35-40% of citrus plants across key growing regions in Indonesia.

Conventional management relies on chemical fungicides, which carry environmental and health risks. Consequently, there is a growing interest in developing eco-friendly alternatives, including microbial antagonists. While antagonistic fungi such as *Trichoderma asperellum* and *Gliocladium* sp. have demonstrated efficacy (Agustina et al. 2019), the use of rhizosphere bacteria remains underexplored. Previous studies have indicated that certain bacterial strains, including *Bacillus subtilis*, *Pseudomonas fluorescens*, and *Paenibacillus polymyxa*, exhibit antifungal activity against *L. theobromae* (Che et al. 2015; Sajitha, Maria & Dev 2014). Seven *Pseudomonas* species from the tomato rhizosphere can inhibit the growth of the wilt disease caused by *Ralstonia solanacearum* and produce lipase, protease, and α-amylase enzymes (Mohammed, Oloyede & Odeseye 2020). According to Dwiastuti and Sugiyatno (2018), using a combination of interstocks on three commercial citrus varieties showed that lime scions combined with Volkameriana, Troyer citrange, and Kanci interstocks had the smallest value of disease intensity (0.2 cm). The combination of Japanese citrus (JC) rootstock and *C. nobilis* vr. Siam Pontianak scion had an average rotten value of 5.48 cm in the moderate severity category.

East Kalimantan presents a unique ecological setting, with contrasting dryland and swamp agroecosystems, high humidity, and consistent tropical temperatures (Whitten et al. 2000). These environmental conditions are known to shape the structure of microbial communities in the rhizosphere (Berendsen, Pieterse & Bakker 2012; Coleine et al. 2024) potentially leading to the development of native rhizobacteria with enhanced biocontrol traits (Compant et al. 2005). Studying rhizosphere bacteria from citrus plants in this region not only supports local disease management strategies but also contributes to global efforts in sustainable plant protection (Kumawat, Razdan & Saharan 2022; Yang et al. 2024). This study aims to (1) characterise the diversity of rhizosphere bacteria from citrus plants grown in upland and swamp environments in East Kalimantan, and (2) evaluate their antagonistic potential against *L. theobromae* through *in vitro* assays.

MATERIALS AND METHODS

ISOLATION AND DIVERSITY ANALYSIS OF RHIZOSPHERE SOIL BACTERIA

Soil samples of Siamese orange plants were obtained in early 2023 from two citrus plantations, dry land, and tidal land in Padang Prapat Village, Tanah Grogot District, Paser Regency, East Kalimantan Province, Indonesia. Rhizosphere bacteria were isolated by suspending 25 g of soil samples in 225 mL of 0.85% NaCl solution. The sample suspension was serially diluted from 10^{-1} to 10^{-5}. A soil suspension of 0.1 mL from each dilution was inoculated into a Petri dish and poured with Nutrient Agar (NA) medium. The bacterial culture was incubated at 30 °C for 24 h (Méndez-Bravo et al. 2023; Suharjono & Yuliatin 2022). The number of rhizosphere soil bacteria was determined based on total plate count (TPC), and the diversity was determined based on the Important Value Index (IVI) according to Equation (1) (Nan et al. 2020; Nguyen et al. 2014). Density, frequency, and dominance were respectively determined based on the number of individuals of a species, the proportion of plots in which the species was present, and the total basal area occupied by the species relative to the overall sampled area (Nguyen et al. 2014). The bacteria colony was isolated based on the streak plate method on the NA media and characterised based on colony and cell morphology.

$$IVI = RD1 + RF + RD2 \quad (1)$$

where IVI is the important value index; RD1 is the relative density; RF is the relative frequency; and RD2 is the relative dominance.

HYPERSENSITIVITY TEST OF RHIZOSPHERE SOIL BACTERIA

Each isolate of rhizosphere soil bacteria was assessed for pathogenicity on tobacco leaves according to the Hypersensitivity (HR) test. One loop of bacterial isolate was inoculated into 50 mL of Nutrient Broth (NB) media and incubated at 30 °C for 48 h. A culture suspension of as much as 0.1 mL of each bacterial isolates with a cell density of 10^7 cells/mL was injected into the underside of tobacco leaves. Tobacco plants were used as hypersensitivity test materials because they exhibit a distinct and easily observable hypersensitive response (HR) when exposed to specific pathogens. This response, characterised by localised cell death and a water-soaked appearance, allows researchers to easily identify pathogenic bacteria (Umesha et al. 2008). The spore suspension of *L. theobromae* and sterile distilled water were also injected into tobacco leaves as positive and negative controls, respectively. Hypersensitivity reactions are indicated by the appearance of necrotic leaf tissue around the injection site after 24-48 h of inoculation (Kesaulya, Virgowati & Celvia 2017).

SCREENING OF RHIZOSPHERE SOIL BACTERIA AGAINST *L. theobromae*

The antagonistic activity of each bacterial isolate against *L. theobromae* was determined based on the double culture technique (Stracquadanio et al. 2020) using PDA media. Each treatment was repeated three times, and the culture was incubated at room temperature (28 ± 2 °C) for 48 h. Radial growth of the pathogen fungus colony was measured after 7 days of incubation time. The inhibition value of bacterial isolate against pathogen fungus was calculated using Equation (2) (Janatiningrum & Lestari 2022). Bacterial isolates with an inhibition value of more than 50% to inhibit the growth of *L. theobromae* were used for further assay.

$$PIRG\ (\%) = (R1 - R2) / R1 \times 100 \qquad (2)$$

where PIRG is the percentage of radial growth inhibition; R1 is the growth radius of pathogenic fungi without bacterial treatment (control); and R2 is the growth radius of the pathogenic fungi with bacterial treatment.

BIOASSAY OF RHIZOSPHERE BACTERIAL ISOLATE AGAINST *L. theobromae*

The antagonist test of potential bacteria isolated against *L. theobromae* was carried out using the disc diffusion method. One loop of each potential bacterial isolate, T4, T13, and T14, was inoculated into 100 mL NB media and incubated in a shaker incubator at 30 °C for 18 h (at logarithmic growth phase) and 48 h (at stationary growth phase). One spore of *L. Theobromae* pathogen fungus was inoculated into the centre of Potato Dextrose Agar (PDA) media, and incubated at room temperature (28 ± 2 °C) for 7 days. One germinated spore of *L. theobromae* fungus was inoculated on the surface centre of PDA media in the Petri dish without antibiotics. Each bacterial culture in the logarithmic and stationary phases with an OD600 value of 0.6 was inoculated on a blank disc and placed in the Petri dish at 22.5 mm from the germinating spore of *L. theobromae*. The cultures were incubated at 30 °C for 7 days, and the fungus colony diameter was measured each day using a digital caliper (Figure 1). The inhibition value of each bacterial isolate against the *L. theobromae* was calculated using Equation (2) (Janatiningrum & Lestari 2022).

EVALUATION OF THE ANTAGONIST MECHANISM OF BACTERIAL ISOLATES AGAINST *L. theobromae*

The *L. theobromae* mycelium at the edge of the clear zone treatment by bacterial isolate T4 and the mycelium of *L. theobromae* (control) from 7 days of incubation (highest inhibition value) were picked up by needle (Torres et al. 2016). Each mycelium sample was placed on a cover glass and coated using platinum, which was carried out using a Coating Chamber tool (Quarum Q150R S) for ± 5 min and left under vacuum for ± 1 min before being removed. The mycelium sample was observed using a Hitachi TM3000 SEM at 2500× magnification.

EFFECT OF pH MEDIA ON THE GROWTH OF POTENTIAL BACTERIAL ISOLATES

One loop of potential bacterial isolates, T4, T13, and T14, was grown in 10 mL NB media. The bacteria culture was incubated at 30 °C for 24 h. The cell density of each bacteria culture was equalised to the OD value of 0.6 (Suharjono & Yuliatin 2022). As much as 2 mL of bacteria cell suspension was inoculated in 18 mL of NB media with different pHs (pH 3, pH 4, pH 5, and pH 7 as a control). The bacteria culture was incubated at 30 °C for 48 h, and the OD value of each culture was measured before and after the incubation period.

IDENTIFICATION OF SELECTED BACTERIAL ISOLATES BASED ON 16S rDNA SEQUENCE SIMILARITY

Genomic DNA of potential bacterial isolates was extracted using the Zymo-SpinTM Kit (Quick-DNATM Fungal/Bacterial Miniprep Kit). The DNA concentration and purity were measured using a nanodrop spectrophotometer. The 16S rDNA sequence was amplified using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The composition of the 50 µL PCR Mix solution consisted of 19 µL ddH2O, 25 µL My Taq Master Mix RED, 2 µL primer 27F (10 pmol/µL), 2 µL primer 1492R (10 pmol/µL), and 2 µL DNA template. The PCR program used is initial denaturation (94 °C for 5 min), followed by 35 cycles consisting of denaturation (94 °C for 30 s),

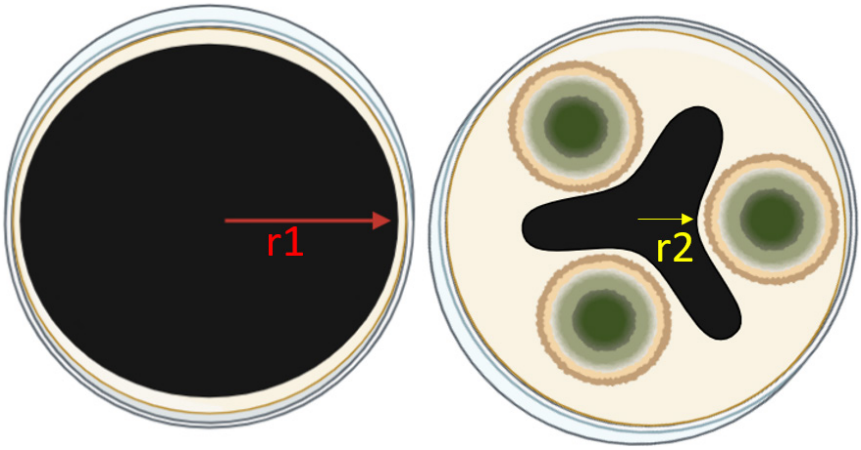


FIGURE 1. Illustration of antagonist assay

annealing (55 °C for 30 s), extension (72 °C for 90 s), and final extension (72 °C for 5 min) (Suharjono & Yuliatin 2022). The 16S rDNA amplicon was purified and sequenced at First Base, Malaysia. The 16S rDNA sequences of bacterial isolates were aligned with reference strains from BLAST and analysed using MEGA 11 software. The phylogeny tree was constructed with the phylogeny tool Bootstrap 1000, and the evolutionary distance method (Neighbor-Joining) was analysed using the Tamura-Nei model (Tamura et al. 2013).

DATA ANALYSIS

The quantitative data from the screening test were analysed using one-way ANOVA, while the antagonistic test data were evaluated through two-way ANOVA. This statistical approach was employed to assess treatment interactions with a 95% confidence level, followed by Tukey post-hoc test. The analysis aimed to determine the differences in inhibition potency of each rhizosphere bacterial isolate against *L. theobromae*, utilizing SPSS version 24 for Windows.

RESULTS AND DISCUSSIONS

MORPHOLOGICAL CHARACTERISATION OF RHIZOSPHERE BACTERIA

There were 17 isolates of rhizosphere bacteria isolated from citrus plants in dry and tidal land. These isolates had different colony and cell morphology (Table 1); 14 isolates (82.35%) were Gram-positive, and three were Gram-negative (17.65%). All isolates had a bacilli cell shape, and only one isolate (T11) was coccus.

DIVERSITY AND ABUNDANCE OF RHIZOSPHERE BACTERIA IN SIAMESE CITRUS PLANTATIONS

Rhizosphere soil bacteria of Siamese citrus were isolated from four locations: acid land block A, dry acid land block B, tidal land block A, and tidal land block B. A number of 17 rhizosphere soil bacteria were obtained from those four locations. Among those bacterial isolates, T4 and T6 had the highest important value index in each location of Siamese citrus plantations (Figure 2). Those two isolates had an important role in those locations. Rhizosphere bacterial associations benefit the plant growth and are known as plant growth-promoting rhizobacteria (PGPR) (Bais et al. 2006; Chamkhi et al. 2022). PGPR can support plant growth by producing growth hormones, dissolving dissolved phosphate, and chelating siderophores, and they can be used as a biocontrol for plant pathogenic bacteria and fungi. PGPR has been widely developed because it is abundant in nature and easy to obtain. The number of rhizosphere bacteria isolated in tidal/swamp land was greater than in dry land (Figure 1). This is due to the nutritional content of tidal land being greater than that of dry acid lands, such as the content of organic matter and organic carbon. Bacterial abundance, diversity and community composition are closely related to characteristics such as pH, carbon-to-nitrogen ratio, carbon, nitrogen, NH_4^+-N, and nutrient availability (Luo et al. 2023).

HYPERSENSITIVITY OF RHIZOSPHERE BACTERIA ISOLATES ON THE TOBACCO PLANT

Based on hypersensitivity tests carried out up to 5 days after infiltration of bacterial isolates, 17 rhizosphere bacterial isolates did not show hypersensitive reactions on tobacco leaves (Table 2 & Figure 3). This result indicated that all rhizosphere bacterial isolates were not pathogenic on tobacco plants. Meanwhile, the positive control of tobacco leaf injected with *L. theobromae* pathogenic fungus showed necrosis symptoms, and there was no necrosis symptom on tobacco leaves at negative control (tobacco leaves without *L. theobromae* injection). According to Kesaulya, Virgowati and Celvia (2017), the hypersensitivity test is declared positive if tobacco leaves infiltrated with pathogenic bacteria produce a localised necrosis response. Thus, the bacteria are said to be pathogenic, and vice versa.

PRELIMINARY SCREENING OF RHIZOSPHERE BACTERIA AGAINST *L. theobromae*

Based on preliminary screening, 17 isolates of rhizosphere bacteria were able to inhibit the growth of *L. theobromae.* The results showed that T4, T13, and T14 isolates have the highest potency (inhibition value > 70%) to inhibit the growth of pathogenic fungus (Figures 4 & 5). According to Živković et al. (2010), the inhibition value was grouped into four categories: low (1-25%), medium (26-50%), high (51-75%), and very high (76-100%). Based on the bioassay result, there were 12 isolates that had low inhibitory values, two isolates that had medium inhibitory values, T4 and T14 that had high inhibitory values, and T13 isolates had very high inhibitory values. According to Otten, Bailey and Gilligan (2004), the antagonist isolates with an inhibitory value of more than 50% could be used as a biological agent to control pathogenic microbes. The T4, T13, and T14 isolates with more than 70% inhibitory values were chosen for further assay against *L. theobromae*.

THE POTENCY OF RHIZOSPHERE BACTERIA ISOLATES TO INHIBIT THE GROWTH OF PATHOGENIC *L. theobromae*

The three potential bacteria isolates (T4, T13, and T14) were grown in the NB medium until logarithmic and stationary phases. Each growth phase of the bacteria isolate was assayed against *L. theobromae*. The results showed that all isolates at the logarithmic growth phase with an incubation time of 1 day to 7 days had higher potency than the control to inhibit the growth of pathogenic fungus *L. theobromae* ($p<0.05$) (Figure 6(A)). The inhibitory value of each bacterial isolate and incubation time of 2 days to 7 days was not significantly different ($p>0.05$). Those bacteria isolated at incubation time over two days had an inhibition value of 70 - 75% against *L. theobromae*.

TABLE 1. Characteristics of colony and cell of citrus plant rhizosphere bacteria

Phenotype characteristic	Bacteria isolate																
	T1	T2	T3	T4	T6	T9	T11	T12	T13	T14	T20	T21	T22	T23	T24	T25	T26
Colony rounded	+	+		+		+	+	-	-	+	-	+	-	+	+	+	-
Colony irregular	-	-	+	-	+	-	-	+	+	-	-	-	+	-	-	-	+
Colony filamentous	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Colony edge, thorough	+	+	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-
Colony edge, flowing.	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	+
Colony edge comprehensive	-	-	-	-	+	+	+	+	+	+	+	+	-	-	+	+	-
Colony elevation, convex	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	-	+
Colony elevation, umbonate	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+	-
Colony elevation, con. wrinkled	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Colony texture, contoured	+	+	+	+	+	-	+	+	-	-	+	+	+	+	+	+	+
Colony texture, radial border	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-
Colony texture, wrinkled	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Colony optical, iridescent	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Colony optical, opalescent	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Colony colour, cream	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Colony colour: white	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
Cell wall, Gram-positive	+	+	+	+	+	+		+	-	-	+	+	+	+	+	+	+
Cell wall, Gram-negative	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-
Cell shape, bacilli	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Cell shape, coccus	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-

+ the bacteria isolate the test character; while - the isolate has no test character

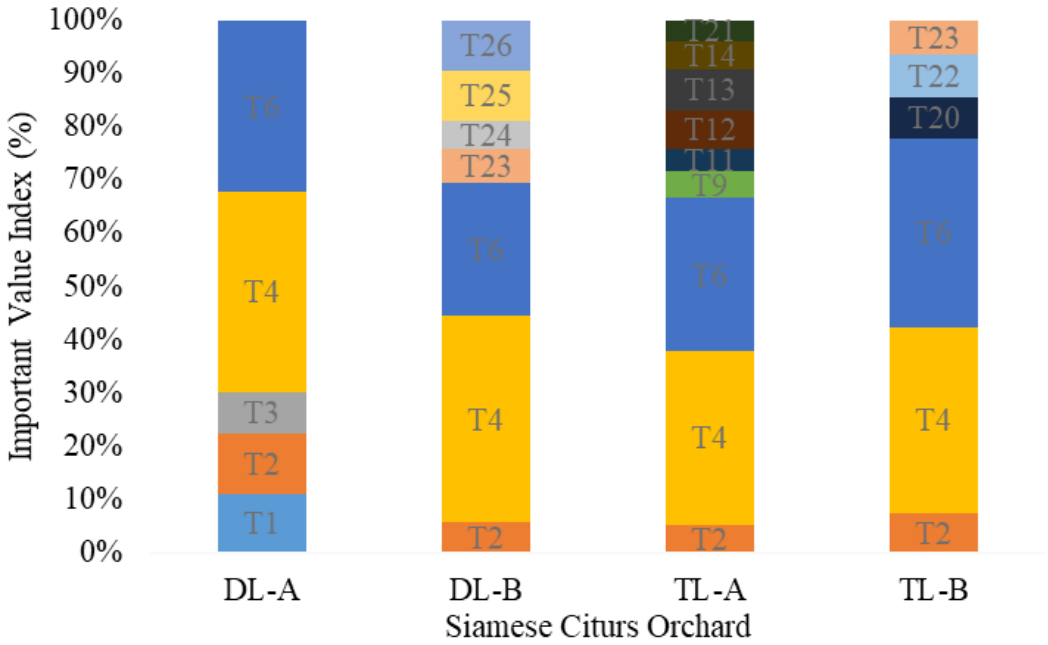


FIGURE 2. Important Value Index (IVI) of rhizosphere bacteria of Siamese citrus plants. DL-A: dry acid land Block A, DL-B: dry acid land Block B, TL-A: tidal land Block A, and TL-B: tidal land Block B

TABLE 2. Hypersensitive activity of rhizosphere bacteria on tobacco leave

Isolate	Hypersensitivity
Negative control	-
T1	-
T2	-
T3	-
T4	-
T6	-
T9	-
T11	-
T12	-
T13	-
T14	-
T20	-
T21	-
T22	-
T23	-
T24	-
T25	-
T26	-

- the bacteria isolate did not induce necrosis of tobacco leaves

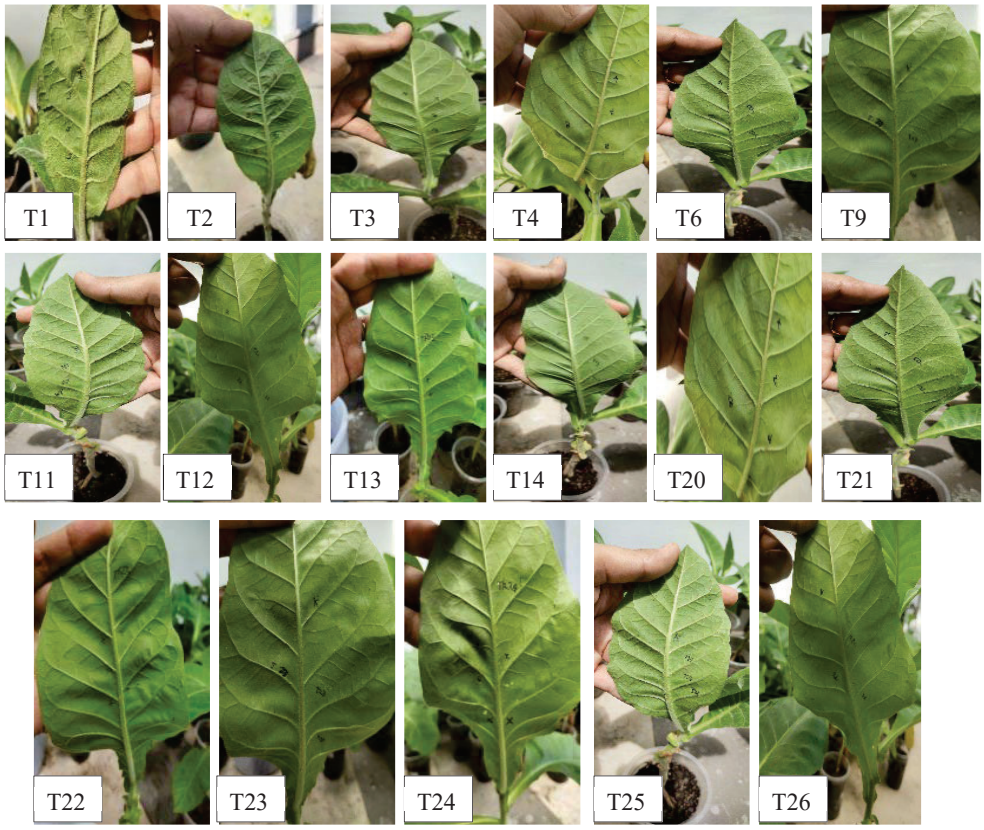
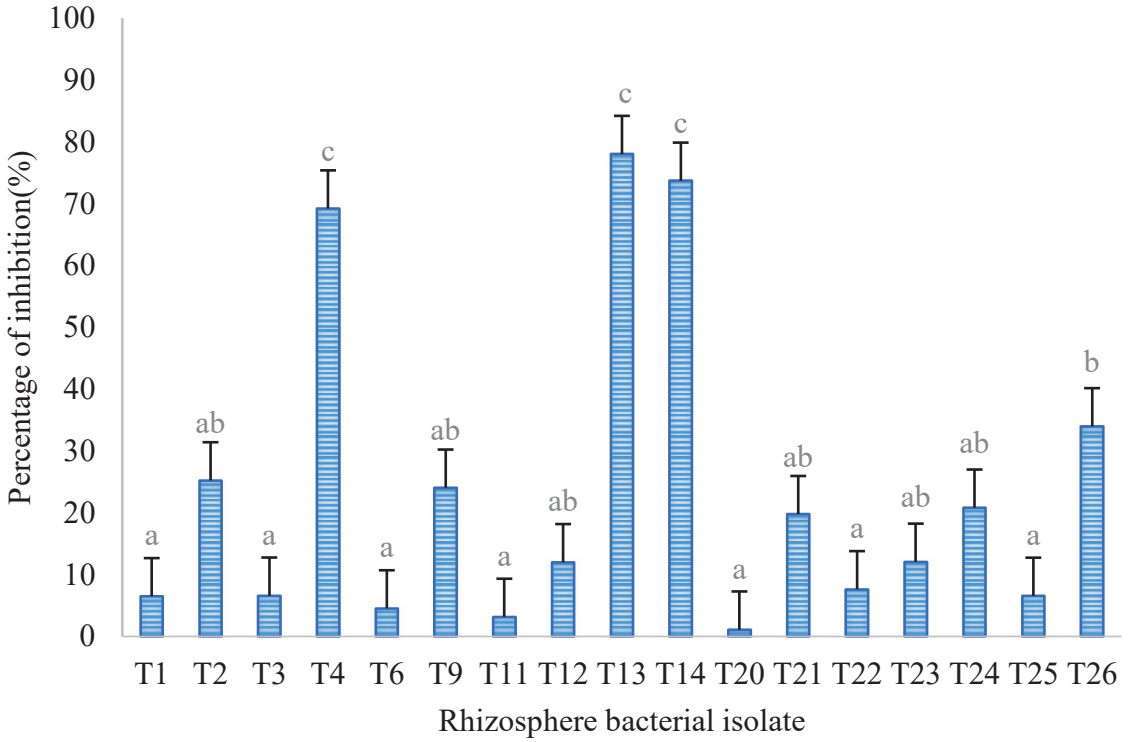


FIGURE 3. Hypersensitivity of rhizosphere bacteria on tobacco leaves



Different notations on the histogram indicate significantly different values among treatments ($p<0.05$)

FIGURE 4. Inhibition value of rhizosphere bacteria against *L. theobromae*

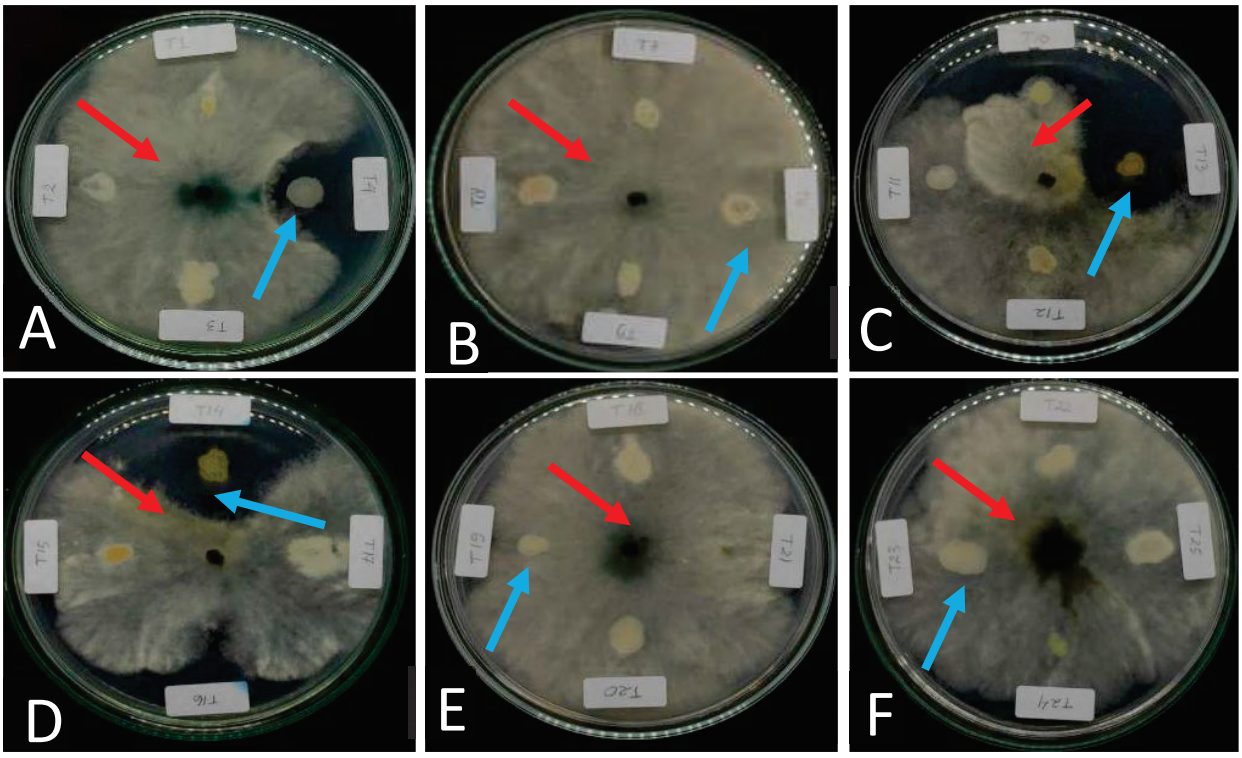
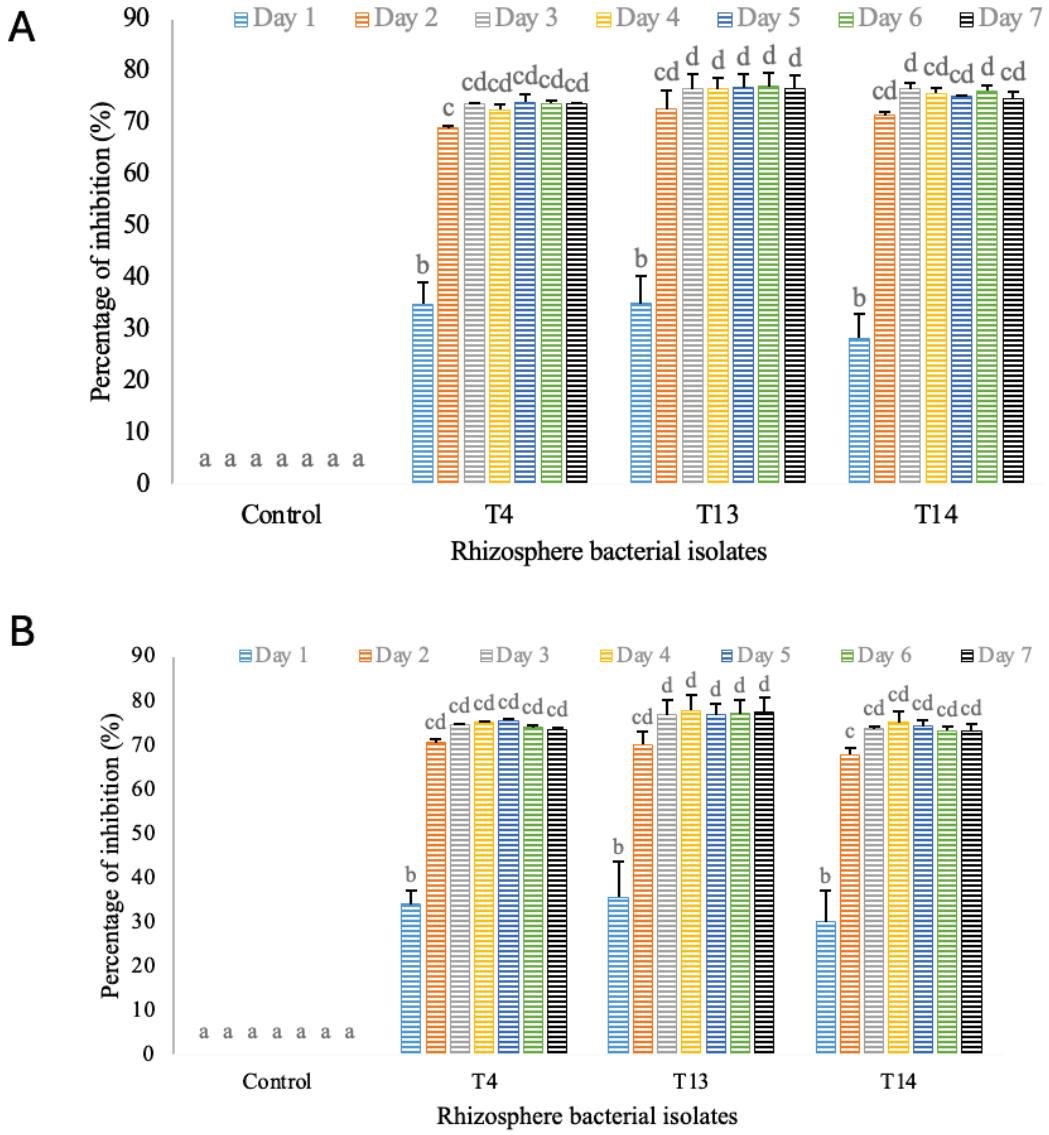


FIGURE 5. Inhibition activity of rhizosphere bacterial against *L. theobromae* on preliminary screening. A, C, and D: bacterial isolates of T13 and T14 T4 with inhibition zone; B, E, F: bacterial isolates without inhibition zone; red arrow: *L. theobromae*, blue arrow: rhizosphere bacterial isolate



Different notations on the histogram indicate significantly different values among treatments ($p<0.05$)

FIGURE 6. The potency of bacterial isolate at (A) the logarithmic growth phase and (B) the stationary growth phase to inhibit the growth of pathogenic *L. theobromae*

Those bacterial isolates showed a clear inhibition zone on the Petri plate inoculated with *L. theobromae* (Figure 7(A)). Inhibition of the pathogenic fungus can be caused by primary metabolites produced by bacterial culture. In the logarithmic growth phase, bacteria grow and multiply twice as fast, and the energy required is higher than in the previous/lag phase. Santamaria et al. (2022) stated that primary metabolite activity goes along with the microbial growth phase and experiences an optimal increase at the end of the logarithmic growth phase or the beginning of the stationary phase. It decreases as microbial activity decreases and nutrients as a substrate are reduced. In the logarithmic growth phase, primary metabolite compounds are generally produced, namely lactic acid, acetic acid, and hydrogen peroxide. Those primary metabolites had toxic activity against other microorganisms.

The results of the antagonist test for potential bacterial isolates at the stationary growth phase had inhibitory values similar to those of bacteria at the logarithmic growth phase. Those bacteria in the stationary growth phase had inhibition values higher than the control ($p<0.05$) to inhibit the growth of pathogenic *L. theobromae* at incubation from 1 day to 7 days (Figure 6(B)). The inhibition value was not significantly different ($p>0.05$) among isolates and among incubation times from 2 days to 7 days of incubation. Those potential isolates at incubation for more than one day had 70% - 80% inhibition values against pathogenic *L. theobromae*. The potential of bacterial isolate at the logarithmic growth phase also produced an inhibition zone against the growth of the potency of bacterial isolate at the logarithmic growth phase to inhibit the growth of pathogenic *L. theobromae* (Figure 7(B)). Those isolates at

the stationary growth phase produce secondary metabolites that are not involved in cell growth, development, or reproduction. Secondary metabolites are low molecular weight compounds secreted by microbes that function as competitive agents against other microbes and act as reproductive hormones and symbiotic agents. In the stationary growth phase, bacterial cell size decreases even though nutrients have been reduced (Kumar, Tiwari & Srivastava 2010). During this phase, a significant amount of secondary metabolites is synthesised as the bacteria defend themselves for survival by releasing these compounds. Some organisms are adversely affected due to changes in environmental conditions caused by the production of these metabolites (Brooks 2007).

Previous research reported that *Bacillus* spp. (Chukeatirote, Phueaouan & Piwkam 2018b; Chukeatirote et al. 2018), *Trichoderma* spp. (Golam & Ilag 1999; Li et al. 2022), and *Pichia* and *Saccharomyces* spp. (Alberto et al. 2022; Mohamed & Saad 2009) are the best biological control agents. *B. velezensis* and *B. amyloliquefaciens* from the rhizosphere of longan plants were able to inhibit 57.87% to 77.32% of the growth of pathogenic *L. theobromae* (Chukeatirote et al. 2023). *Bacillus's* biocontrol of plant diseases involves various mechanisms, such as antibiosis, competition for nutrients, and triggering defence responses in the host plant (Panneerselvam et al. 2019). Several studies showed the antagonistic activity of *Bacillus* against various fungal pathogens, including *L. theobromae* (Chukeatirote, Phueaouan & Piwkam 2018; Chukeatirote et al. 2018; Zhou et al. 2021).

P. aeruginosa Rambhas-2 (PaRS) had an inhibition value of 73.70% against the growth of pathogenic *L. theobromae* (Waghunde & Sabalapra 2015). The mechanism by which PaRS inhibits pathogens can be either direct or indirect, involving the production of secondary metabolites such as antibiotics and hydrogen cyanide. *Pseudomonas* spp. inhibit pathogens through the synthesis of cell wall-degrading enzymes or by the production of secondary metabolites like 2,4-diacetylphloroglucinol, phenazine-1-carboxylic acid, and pyrrolnitrin (Mercado-Blanco & Bakker 2007).

ANTAGONISM MECHANISM OF POTENTIAL BACTERIAL ISOLATES AGAINST PATHOGENIC *L. theobromae*

The bioassay results (Figure 7) suggested that those bacterial isolates had antibiosis activity against pathogenic fungus. Those bacterial isolates had an inhibition value of 77.56% to suppress the growth of *L. theobromae*. Mycelium samples of *L. theobromae* were taken from double culture plates at the border of the inhibition zone and examined using SEM. The mycelium of *L. theobromae* without rhizosphere bacteria treatment grew abundantly, normally, healthily, and was intact (Figure 8(A), 8(C)). The mycelium structural of *L. theobromae* treated with the T13 antagonist bacterial isolate underwent deformation (with wrinkles on the surface) and then ruptured, indicating loss of turgidity and cellular content (Figure 8(B), 8(D)). Morphological alterations in the mycelia of pathogenic fungi following treatment with antagonistic bacteria may be attributed to the metabolites and degrading enzymes produced by the antagonistic bacteria. This mechanism of antagonism has been documented in various fungal pathogens. For instance, when *Aspergillus* mycelia were exposed to *Pseudomonas* and *Bacillus* bacteria, morphological abnormalities, such as deformation and swelling of the mycelia, were observed (Mardanova et al. 2017), and this might be caused by several cell wall degrading enzymes produced by the two bacteria.

Our results indicated that isolated rhizobacteria had antifungal (antibiosis) activity against *L. theobromae* and can be used in biocontrol applications. This potential needs to be investigated further, especially to identify the active components in the culture supernatant of isolate T13. The inhibition of pathogenic fungal growth and the resulting hyphal damage may be attributed to the secretion of hydrolytic enzymes and lipopeptides by antagonistic bacteria (Gong et al. 2015; Li et al. 2015).

THE POTENCY OF RHIZOSPHERE BACTERIA ISOLATES TO INHIBIT THE GROWTH OF *L. theobromae* AT SEVERAL pH CULTURE MEDIUMS

Based on growth tests at several pH mediums, it is known that the three bacterial isolates have different growth abilities. T4 and T13 could grow in the culture medium at pH 5 and 7, respectively, while the T14 isolate could grow at pH 4. The bacterial isolates T4 and T13 had the best growth at pH 5 and 7, whereas the T14 isolate had the best growth at pH 5. Isolates T4 and T13 were unable to grow in the culture medium at pH 3 and 4, while isolate T14 could only grow at pH 3 (Figure 9). The bacteria can be said to be growing if there is an increase in OD600 of at least 0.6. Based on the results of this pH test, these three bacterial isolates can be used in different field pH conditions according to their respective capabilities.

POTENTIAL OF RHIZOSPHERE BACTERIA AS BIOLOGICAL CONTROL AGENTS FOR PATHOGENIC FUNGUS *L. theobromae* IN CITRUS PLANTS

Three isolates which have potential antagonistic agents to inhibit the growth of pathogenic fungus *L. theobromae* were identified based on 16S rDNA similarity. The isolate T4 was identified as *Bacillus subtilis*, with 99.37% similarity to *B. subtilis* DK15 (Figure 10). This identification result was in agreement with the results from Ezrari et al. (2021), which has identified 20 citrus rhizosphere bacteria with important antifungal activity against *F. solani.* Several genera have been identified, namely *Bacillus* (16) (*B. subtilis* (4), *B. halotolerans* (4), *B*. a*myloliquefaciens* (4), *B. licheniformis* (1), *B. velezensis* (1), *B. xiamenensis* (1), and *B. tequilensis* (1)), *Stenotrophomonas* (3) (*S. maltophilia* (2) and *Stenotrophomonas* sp.) and *Sphingobacterium multivorum* (1).

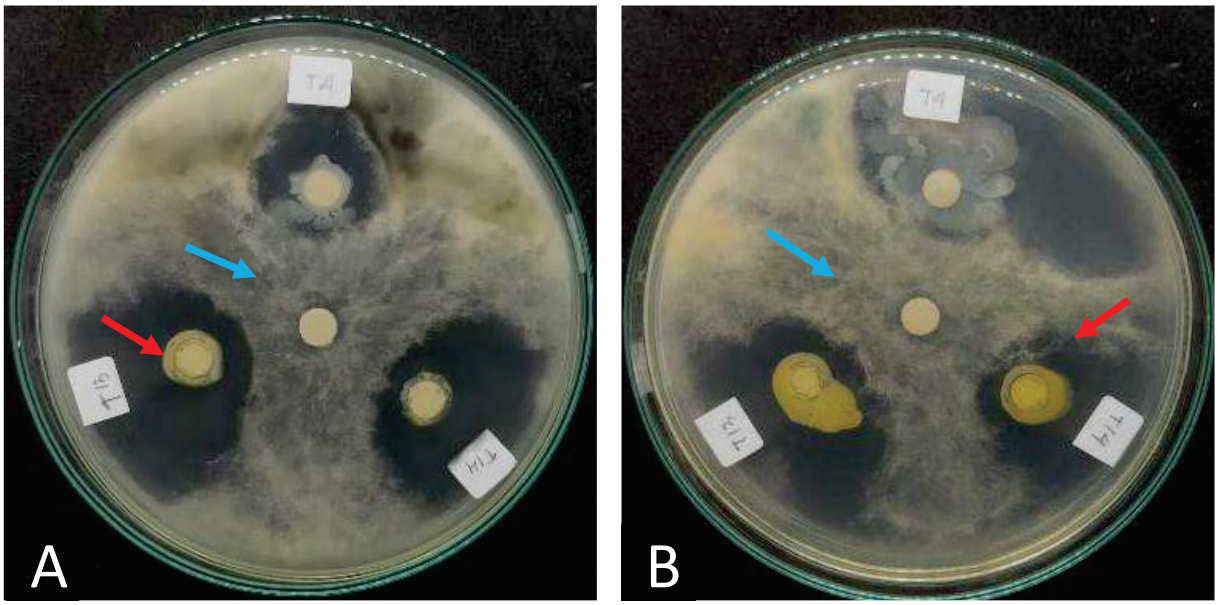


FIGURE 7. Inhibition activity of rhizosphere bacteria against pathogenic *L. theobromae* (A) Logarithmic growth phase; (B) Stationary growth phase; red arrow: *L. theobromae*, blue arrow: rhizosphere bacterial isolate

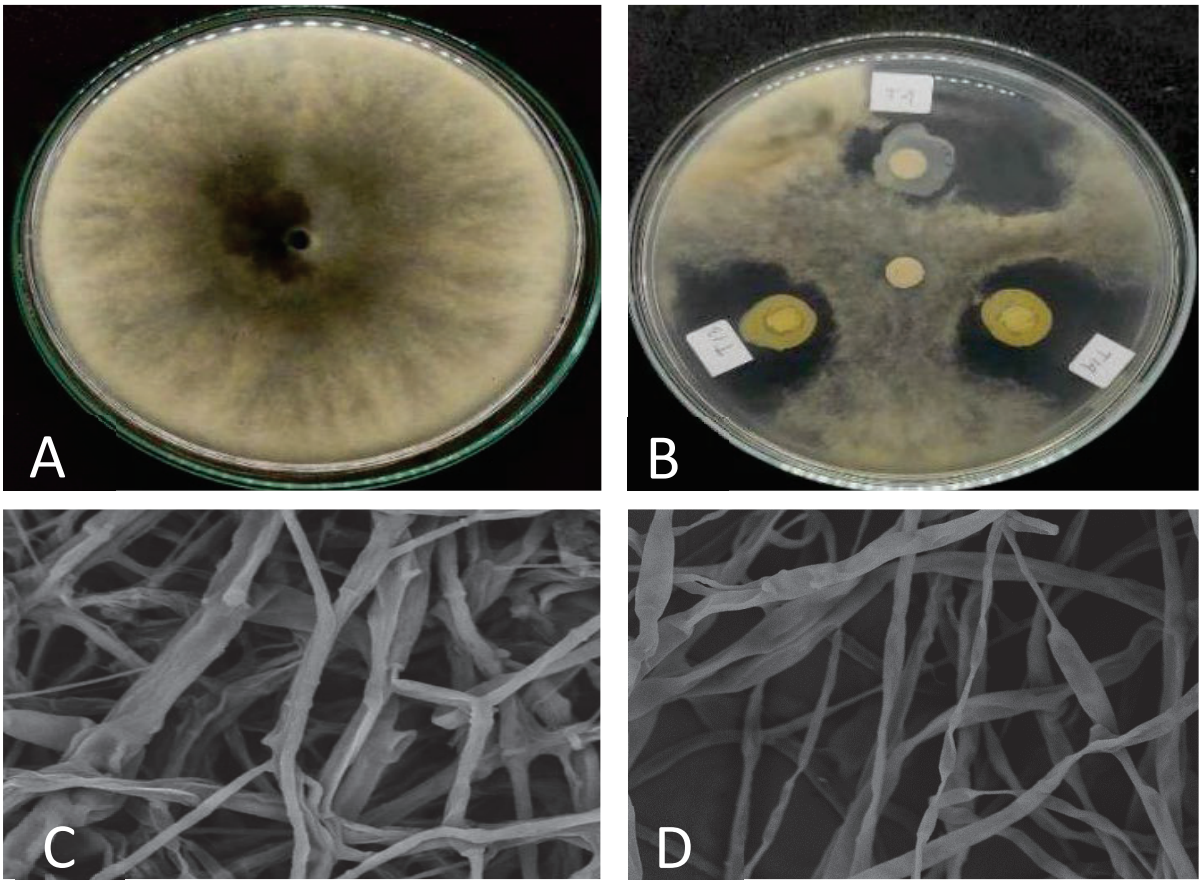
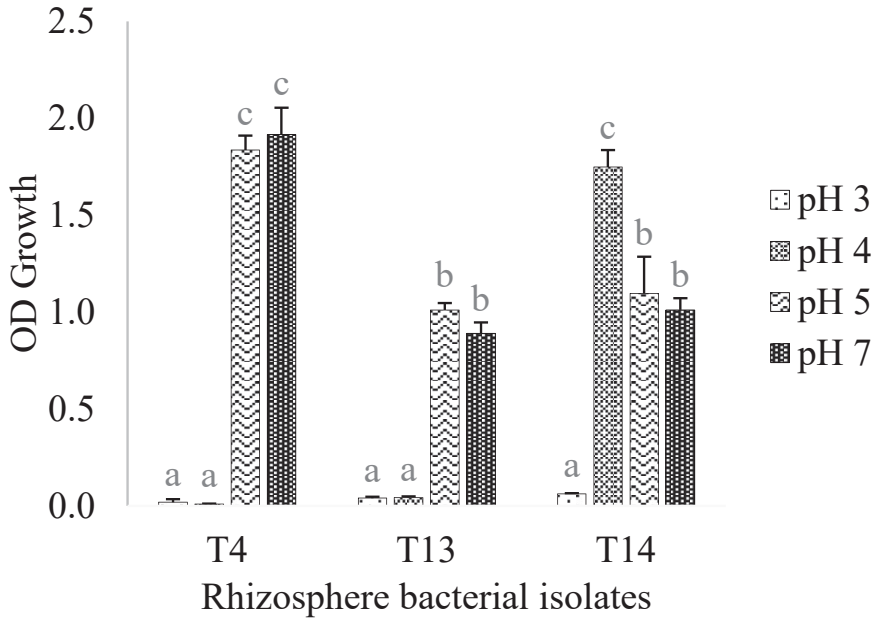
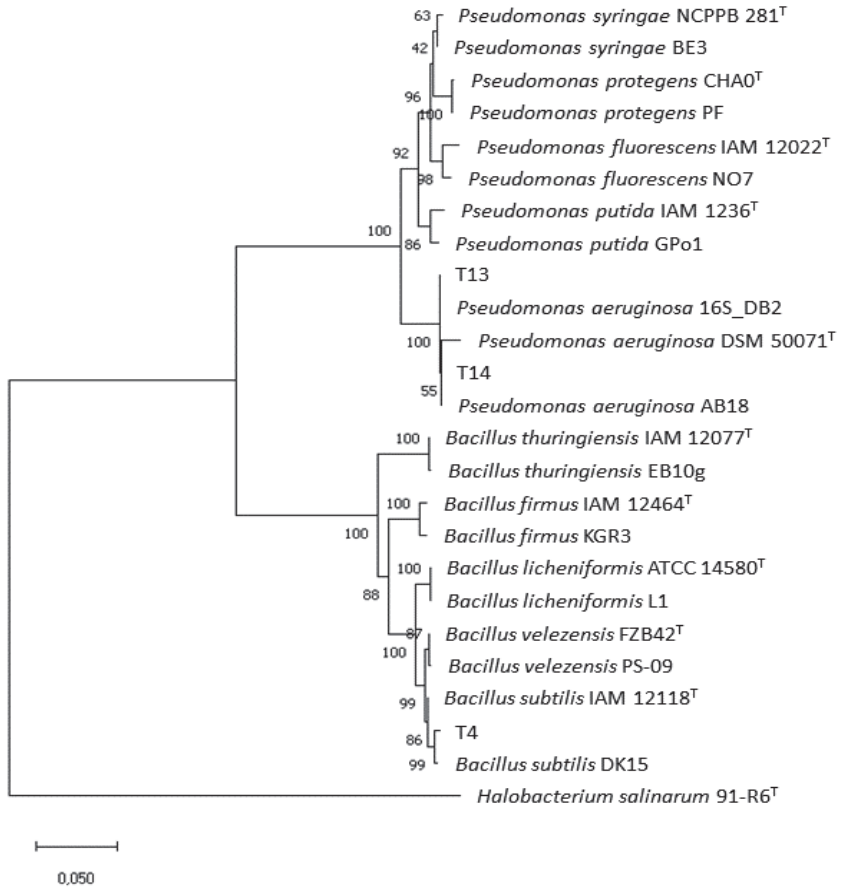


FIGURE 8. Antagonism mechanism of rhizosphere bacteria against pathogen *L. theobromae.* The culture of *L. theobromae* control (A), the culture of *L. theobromae* with bacteria isolates (B); Mycelium structure of *L. theobromae* control (C): Mycelium structure of *L. theobromae* treatment by bacteria isolates (D)



Different notations on the histogram indicate significantly different pH values among treatments ($p<0.05$)

FIGURE 9. Growth of potential antagonistic bacteria at different pH culture media



The number at each branch shows the bootstrap value of the branch

FIGURE 10. Phylogeny tree of isolates T4, T13, and T14 with reference species based on 16S rDNA sequence similarity

The isolates T13 and T14 were identified as *P. aeruginosa* with 99.86% similarity to *P. aeruginosa* 16S DB2 and 99.93% to *P. aeruginosa* AB18, respectively (Figure 9). Citrus rhizosphere bacterial isolates consist of *Pseudomonas, Agrobacterium, Rhizobium, Cupriavidus, Bradyrhizobium, Mesorhizobium, Cellvibrio, Sphingomonas, Variovorax*, *Paraburkholderia*, and *Burkholderia,* some of which are potential microbes that are beneficial to plants (Xu et al. 2018). The consortium of various citrus rhizobacterial species is related to the diversity found in citrus root exudates, which can attract these species and support their growth to colonise citrus root tissue (Chenniappan et al. 2019; Díaz et al. 2016).

Based on these findings, *B. subtilis* (T4) and *P. aeruginosa* (T13 and T14) from the rhizosphere of citrus plants showed strong antagonistic potential against *L. theobromae*, which causes stem rot disease in citrus plants. Several authors have reported that three *Bacillus velezensis* strains, YK194, YK201, and YK268, with better antagonistic effects and high stability against *L. theobromae* were isolated from the rhizospheric soil of healthy avocado plants. Further investigation on the mechanism of action of antagonistic strains showed that *B. velezensis* YK268 could produce lipopeptides, namely, surfactin, fengycin, and iturin, which could significantly inhibit the spore germination of *L. theobromae* (Xiaoyu et al. 2024).

Previous reports have suggested that *B. subtilis* and *B. amyloliquefaciens* are able to inhibit the growth of *L. theobromae* (Arrebola, Jacobs & Korsten 2010; Sajitha, Maria & Dev 2014), and in the latter case, the lipopeptide iturin A was identified as the principal inhibitor of many fungal pathogens (Arrebola, Jacobs & Korsten 2010). The isolate named JN15 showed maximum inhibition of the fungus *L. theobromae*, approximately 60% (Ekachai, Thanong & Anong 2018). Bacteria from the rhizosphere of different fruit trees in Morocco were tested for their potential to inhibit causal agents of trunk diseases in apple trees, including *L. theobromae*. Genus Bacillus PH34Z5 showed strong antagonistic activity against *L. theobromae* (94.12% inhibition). Most of these bacterial strains secreted hydrolytic enzymes that can degrade fungal cell walls. In plant growth promotion assays with *Brassica napus* seedlings, the selected bacteria, particularly strains PH1Z8 and PM6Z12, enhanced plant growth compared with the negative controls (Khadija et al. 2025).

CONCLUSION

There were 17 isolates of rhizosphere bacteria isolated from citrus plants in dry and tidal orchards in East Kalimantan Province; all isolates had no potential as plant pathogens. There are more rhizosphere bacteria in swamp orchards than in acid-dry land. Three rhizosphere bacterial isolates, T4, T13, and T14, could potentially inhibit the growth of the citrus plant pathogen *L. theobromae*. The T13 isolate had a high potency in inhibiting pathogenic fungus growth, and it could grow at pH 5 and 7. The T4 isolate was identified as *B. subtilis*, while the T13 and T14 isolates were identified as *P. aeruginosa.* Those bacterial isolates can be developed as biological control agents to suppress the pathogenic fungus of the citrus plants.

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