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Isolation and Molecular Identification of a Siderophore Producing Bacterium and its Antagonistic Effect Against *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4

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ABSTRACT

Bananas are one of the world's most consumed fruits. Developing countries in the Global South depend on bananas for food security and livelihoods. Still, the banana industry also drives a multinational trade worth billions of US dollars. In addition, banana plants also hold cultural and religious significances in many Asian countries. However, banana production faces several challenges, and one of the major issues is the Fusarium wilt disease caused by the fungus *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (Foc TR4). This disease threatens numerous banana cultivars, including the Cavendish, the most traded banana cultivar. Therefore, the objective of this study was to find effective measures to control the spread of this disease through antagonistic soil bacteria. This study isolated 14 fluorescent, siderophore-producing bacteria with *in vitro* inhibition rates of 21.73-50.38% against Foc TR4 from the soil surrounding banana plants. Most of the isolates were identified as *Pseudomonas* spp. via 16S rRNA sequencing, and phylogenetic analysis based on 16s rRNA sequences showed clustering of isolates congruent with the results of similarity searches. Three isolates that exhibited relatively higher antagonistic activity against *Fusarium oxysporum* f. sp. *cubense* compared to other isolates were JBAA132 (50.38%), K2B131 (46.28%), and KTP231 (45.38%). Isolate JBAA132 displayed differences in biochemical characteristics compared to its closest match (*Pseudomonas aeruginosa*

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type strain DSM50071). It also demonstrated nitrogen-fixing and phosphate solubilising properties common to plant growth promoters. Therefore, isolate JBAA132 may be further explored as a potential biocontrol agent in curbing the spread of Foc TR4.

Keywords: Biological control, Foc TR4, Fusarium wilt, Panama disease, plant growth promoter, *Pseudomonas aeruginosa*, siderophore

INTRODUCTION

Bananas are cultivated in more than 135 countries and are a staple food for many developing nations in Africa and Asia. Banana production was valued at 38 billion USD in 2018 and had an export value of 14.7 billion USD in 2020 (Workman, 2021). However, the production of bananas is severely threatened by Panama disease, also known as Fusarium wilt, caused by the fungus *Fusarium oxysporum* f. sp. *cubense* (Foc) (ProMusa, n.d.). Foc is classified into distinct races according to the banana varieties they infect, namely races 1, 2, 3, and 4. Foc Race 1 affected the Gros Michel banana and decimated plantations across Central America during the 1950s, prompting farmers to switch to the Cavendish because it was resistant to Foc Race 1 (Ploetz, 2005). However, a new and more virulent strain of Foc emerged in the 1970s, infecting the Cavendish bananas in Taiwan (Su et al., 1986). This new strain of Foc termed Foc Tropical Race 4 (TR4) can infect *Musa* spp. that is susceptible to Foc Race 1 and Race 2 in addition to the Cavendish. Furthermore, vegetative propagation of commercial banana plants results in daughter plants that are clones of the parent plant and are susceptible to the same disease. Increasing globalisation and the worldwide trading of bananas have also caused the Fusarium wilt to spread rapidly. The losses continue to rise as current prevention and management measures are ineffective and insufficient to control the disease (Scheerer et al., 2018). As such, Foc TR4 poses a serious threat to global banana production and food security (Food and Agriculture Organization of the United Nations [FAO], 2019).

Fusarium oxysporum f. sp. *cubense* TR4 is an ascomycete that reproduces asexually via microconidia, macroconidia, and chlamydospores (Ghag et al., 2015). According to VanderMolen et al. (1987), infection by its spores causes the host plant to form tyloses that gel up the xylems. The occlusion of the xylem prevents the host from transpiration and water transport, eventually killing the plant. Fusarium wilt is extremely difficult to control because the spores are durable and can persist in the soil for up to 30 years (Agrios, 2005; Hennessy et al., 2005). Therefore, finding reliable and efficient prevention methods is crucial for the survival of the banana industry and the livelihoods of communities that rely on banana production. While the development of disease-resistant cultivars is highly desirable, it is costly and time-consuming (Dita et al., 2018; Zuo et al., 2018). On the other hand, fungicides and sterilants have limited efficacy and are considered unsustainable due to the possible adverse

effects on human health and the ecosystem (ProMusa, n.d.). Meanwhile, biological control or biocontrol agents reliant on an organism's natural functions to control a pest or a disease (Vincent et al., 2007) are progressively being investigated for the potential as a sustainable option to curb the spread of Foc TR4 (Sharma et al., 2017; Vincent et al., 2007).

Potential biocontrol candidates come from the genus *Pseudomonas* spp., ubiquitous Gram-negative, motile, flagellated, rod-shaped, and aerobic gammaproteobacteria (Jenny & Kingsbury, 2018; Liao, 2006; Schroth et al., 2018; Weller, 2007). Members of *Pseudomonas* spp. have exhibited suppressive ability towards plant diseases such as the tomato disease caused by *Pythium aphanidermatum* (Jayaraj et al., 2007), pepper blight caused by *Phytophthora capsici* (Sang & Kim, 2014), and pink snow mould of wheat caused by *Microdochium nivale* (Andersson et al., 2012). In addition, some strains of *Pseudomonas* spp. produce fluorescent pigments, such as pyoverdine or pyochelin during iron-deficit conditions, are observable under ultraviolet (UV) light (Budzikiewicz, 1996). These fluorescent pigments are iron chelators known as siderophores that allow the bacteria to scavenge ferric ions (Fe^{3+}) from the surroundings for DNA synthesis, oxygen transport, incorporation into proteins, and nitrogen fixation (Andrews et al., 2003; Cornelis & Matthijs, 2007). Simeoni (1987) found that the germination of *F. oxysporum* f. sp. *cucumerinum* chlamydospores was inhibited when Fe^{3+} concentration in the soil reached 10^{-19} M and Fe^{3+} concentrations of 10^{-22} to 10^{-27} M produced optimal fungal inhibition. Meanwhile, Sayyed and Patel (2011) discovered that siderophore rich broth and supernatants exhibited strong antifungal activity against several types of fungi, including *Aspergillus niger* and *F. oxysporum*. These findings indicated that siderophore production and iron availability is strongly correlated to the antagonistic ability of *Pseudomonas* spp. against fungi.

Therefore, this study aimed to isolate potential biocontrol bacterial agents to suppress Foc TR4 by screening for fluorescent siderophore-producing bacteria and evaluating the antagonistic activities of the isolates against Foc TR4. Isolates exhibiting antagonistic activity were subsequently identified using 16s rRNA sequencing. In addition, the isolate that demonstrated more than 50% *in vitro* inhibition rate against Foc TR4 was further characterised using biochemical assays.

MATERIALS AND METHODS

Isolation and Screening of Fluorescent Isolates

Soil samples were collected from four points (north, south, east, west) at a depth of 20 cm around banana plants from Pahang and Selangor, Malaysia (Table 1). Healthy (N = 44) and affected (N = 11) banana plants were selected randomly to increase the possibility of getting various bacterial samples.

Table 1
List of sampling sites and their corresponding coordinates

Location	Coordinates	State
Felda Lembah Klau, Raub	N3˚41'29.454''; E102˚0'20.214''	Pahang
Sungai Pelek, Sepang	N2˚40'15.4''; E101˚41'22.4''	Selangor
Ladang Kongsi, UPM	N2°58'51.2"; E101°42'45.4"	Selangor
Kolej Kedua, UPM	N2°59'40.6"; E101°42'20.6"	Selangor
Kolej Tun Perak, UPM	N2°59'29.9"; E101°42'26.8"	Selangor
Jabatan Biology, UPM	N3˚0'4.8204''; E101˚42'17.0604''	Selangor

The samples were stored at 4 ˚C and processed two weeks after collection. A modified protocol of Sudarma and Suprapta (2011) and Nawangsih and Purba (2013) was used for screening. First, samples collected from the four points of each plant were mixed, then one gram of the mixed soil sample was taken and diluted in 99 mL of sterilised water to make up to 100 mL. After five minutes of agitation, the suspension of each mixed sample was used to prepare three spread plates on King's B agar (Pronadisa, Condalab, Spain) to isolate fluorescent bacteria, each with 1 mL of suspension. The plates were incubated at 25 ˚C ± 2 for 48 h, then viewed under UV light in an Enduro™ GDS-1302 gel documentation system (Labnet International, USA). Fluorescent colonies were picked and streaked on King's B agar, then incubated at 25 ˚C ± 2 for 24 h.

Determination of Siderophore Production using Chrome Azurol S Overlay

Blue Chrome Azurol S (CAS) dye was prepared according to Pérez-Miranda et al. (2007). A 7:3 mixture of blue CAS agar was prepared by slowly adding the dye to a 1% (w/v) agarose solution, then poured onto the Petri dish containing 24 h colonies of a single isolate, and then incubated at 25 ˚C for 2 h. The development of an orange-yellow zone indicated positive siderophore production. Therefore, colonies with positive siderophore production were chosen for the *in vitro* fungal antagonistic test. An uninoculated filter paper disc (4 mm) in place of bacterial culture was used as a negative control. All experiments were repeated at least twice with three replicates for each isolate.

In vitro Antagonistic Assay

The Foc TR4 (culture collection number: 9888, isolated initially from Fusarium wilt infected banana, Kuala Terengganu, Terengganu) for the antagonistic test was provided by the Fusarium Collection Centre, Plant Pathology Laboratory, Universiti Sains Malaysia (USM). A dual culture *in vitro* antagonistic test was performed. First, a filter paper disc (4 mm) was soaked in 24 h cultures of the isolate, then placed at 5 cm from a mycelial plug (4 mm) of Foc TR4 on

potato dextrose agar (PDA) (Merck, USA). After that, the plate was incubated at 25 ˚C ± 2 for 10 days. This assay was carried out in triplicate. An uninoculated filter paper disc was used as a negative control. The percentage of inhibition was calculated according to the formula provided in Chaiharn et al. (2009).

$$\%\ of\ inhibition = \left(\frac{D_c - D_t}{D_c}\right) x\ 100$$

Where Dc = distance of fungal growth in the control plate when it was cultured opposite a blank disc, and Dt = distance of fungal growth when it was cultured opposite a filter paper disc inoculated with the isolate. A one-way analysis of variance (ANOVA) was performed, followed by Tukey's honest significant difference (HSD) post-hoc test to determine if the inhibition rates were significantly different (GraphPad Prism version 9.2.0 for Windows, GraphPad Software, USA, www.graphpad.com). The isolates were then identified via molecular means.

Molecular Identification of Bacterial Isolates

The DNA of each isolate was extracted using the methods described in Nasiri et al. (2005) with slight modification by substituting the mentioned laundry powder with 25 mg/mL of enzymatic Daia washing powder (PT. Sayap Mas Utama, Indonesia). Amplification of the 16s rRNA region was performed on PCRmax Alpha Cycler (PCR Max, USA) using primers 16SF (5'-CGGTTACCTTGTTACGACTT-3') and 1387R (5'-GCCCGGGAACGTATTCACCG-3') obtained from Nawangsih and Purba (2013), and GoTaq® Flexi DNA Polymerase reagents (Promega, USA). In each 20 µL reaction, it contained 2 µL GoTaq® buffer (5x), 1 µL magnesium chloride (25 mM), 2 µL dNTPs (2 µM), 2 µL of each forward primer (2 µM) and reverse primer (2 µM), 1 µL GoTaq® polymerase (0.5U/µL), 2 µL DNA (10-20 ng), and 8 µL of distilled water. The cycling profile started with an initial denaturation of template DNA at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, annealing at 64 °C for 30 s, elongation at 72 °C for 30 s, and a single cycle of final elongation at 72 °C for 5 min. The PCR products were sequenced using the service provided by Apical Scientific Sdn. Bhd. (Seri Kembangan, Malaysia). The 16s rRNA sequences were searched against the National Centre of Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990), and the Ribosomal Database Project (RDP) using the sequence aligner (Q. Wang et al., 2007). The 16s rRNA sequences of all isolates were deposited into the NCBI database with the accession numbers of MN203661 (JBAA132), MN203662 (K2B121), MN203663 (K2B131), MN203664 (K2B421), MN203665 (K2B431), MN203666 (KTP211), MN203667 (KTP231), MN203668 (SNH212), MN203669 (SNH222), MN203670 (SNH231), MN203671 (SNH232), MN203672 (TGB111), MN203673 (TGB112), MN203674 (TGB131).

Phylogenetic Analysis

The 16s rRNA sequences of the most significant match for each isolate based on Nucleotide BLAST (BLASTn) were retrieved from NCBI and used for phylogenetic analysis in Molecular Evolutionary Genetics Analysis (MEGA)-X (version 10.2.6) (Kumar et al., 2018). An unweighted pair group method with arithmetic mean (UPGMA) tree (Sneath & Sokal, 1963) was constructed with 1,500 bootstrap replicates using the Tamura-Nei model (Tamura & Kumar, 2002) and rooted using *Aquaspirillum polymorphum* NRBC 13961 (NR_104710.1) as an outgroup. Branches corresponding to partitions reproduced in less than 70% of bootstrap replicates were collapsed.

Biochemical Characterisation

The isolate that displayed more than 50% of *in vitro* antagonistic activity and defined inhibition pattern was chosen for further characterisation. Next, the nitrogen-fixing test was performed using the methods explained in Baldani et al. (2014). Meanwhile, the phosphate solubilisation test was conducted based on the protocols provided in Chatli et al. (2008). Lastly, the catalase test, cetrimide test, citrate test, decarboxylase test (arginine, lysine), gelatine liquefaction test, indole production, lipase solubilisation test, Methyl Red-Voges Prokaur (MR-VP), nitrate reduction, oxidase test, oxidative-fermentative test (dextrose, inulin, lactose, mannitol, sucrose), and sulphate-indole-motility test were all performed according to the protocols described in Cappuccino and Sherman (2008).

RESULTS AND DISCUSSION

Isolation, Screening, and Siderophore Production Assay

A total of 55 soil samples were collected from the immediate vicinity of banana plants. From these samples, 32 fluorescent isolates were obtained. However, only 14 of the fluorescent isolates—KTP231, JBAA132, SNH212, K2B421, TGB112, SNH222, SNH232, TGB131, K2B431, KTP211, TGB111, K2B131, SNH231, and K2B121—were positive for siderophore production. Figure 1 shows the differences observed in control a non-siderophore producer and a siderophore producer. The orange halos surrounding the colonies indicate the presence of siderophores.

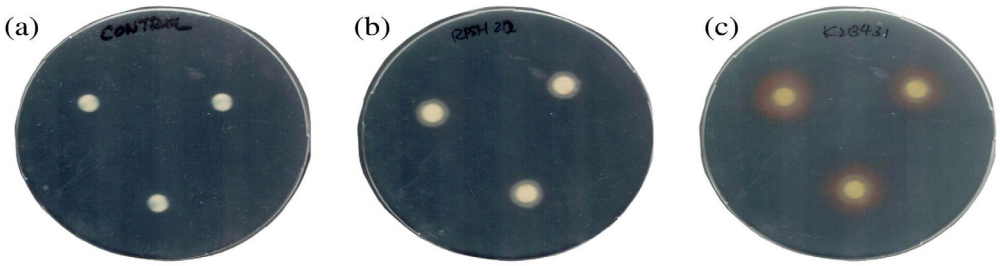

Figure 1. Siderophore production assays. (a) Control plate with uninoculated filter paper discs; (b) plate showing a lack of orange halo around the colonies, thus indicating a non-siderophore producer; (c) plate showing orange halo around colonies, thus indicating a siderophore producer

In vitro Antagonistic Assay

The antagonistic ability of the 14 siderophore producers against Foc TR4 was tested on PDA. All 14 isolates demonstrated various inhibition rates after ten days of incubation. Compared to the control plate, inhibitions were observed in all the plates indicated by the halt of mycelial growth towards the bacterial isolates. Figure 2 shows the Foc TR4 mycelial growth in the control plate (a) and cultured with inhibitive isolates (b-o) after ten days of incubation. The inhibition of the mycelial growth can be clearly seen in plates (b) JBAA132, (c) K2B121, (d) K2B131, (e) K2B421, and (h) KTP231. A less defined inhibition zone was observed in all other plates, with plates (m) TGB111 and (o) TGB131 displaying the weakest inhibition abilities. Among the isolates screened, seven isolates showed more than 40% *in vitro* inhibition rates. The percentages of inhibition for all 14 isolates were plotted and are shown in Figure 3. One-way ANOVA revealed a significant difference in the inhibition rate ($p < 0.0001$). The Tukey's HSD post-hoc test found that the inhibition rates of JBAA132, K2B131, KTP231, SNH212, K2B121, KTP211, SNH232, TGB111, SNH222, TGB112, K2B43, and K2B4211 were significantly different to the control ($p < 0.0001$). However, there was no significant difference among the isolates except for JBAA132 vs. SNH231 and JBAA132 vs. TGB131.

Antagonistic bacteria play an important role in suppressing soil-borne plant diseases and are potential biocontrol agents. The fungal inhibition efficiency of various bacterial species against Foc has been studied over the years. Li et al. (2012) studied 45 isolates of *Pseudomonas* spp. and discovered that these isolates demonstrated an inhibition rate that ranged from 38.30% to 67.14% against Foc. Meanwhile, Yuan et al. (2012) showed that volatile compounds from *Bacillus amyloliquefaciens* achieved 30-40% inhibition in dual culture plates against Foc. Zacky and Ting (2013) discovered that cell and cell-free extracts of *Streptomyces griseus* produced a 54% and 33% rate of inhibition on Foc-TR4. Meanwhile, the usage of *Burkholderia cenocepacia* in Ho et al. (2014) demonstrated an inhibition rate of 44.4% against Foc TR4. Similarly, a study by Islam et al. (2018) demonstrated that *P. aeruginosa* BA5 could inhibit Foc growth at a rate of up to 58.33%. It is plausible that isolate JBAA132 shows a comparable inhibition rate at 50.38% against Foc TR4.

Molecular Identification of Isolates

Molecular identification of the 14 antagonistic isolates using 16S rRNA revealed that all isolates were from the genus *Pseudomonas*, except for isolates KTP211 and SNH231. Similarity searches against both NCBI and RDP databases returned similar significant matches for each isolate. For example, a BLAST search of the 16s rRNA sequence of isolate JBAA132 showed a 99.48% similarity to *Pseudomonas aeruginosa*, followed by *Pseudomonas otiditis* (98.07%) and *Pseudomonas guezennei* (97.96%). Similarity search against RDP also returned *P. aeruginosa* as the top match but only

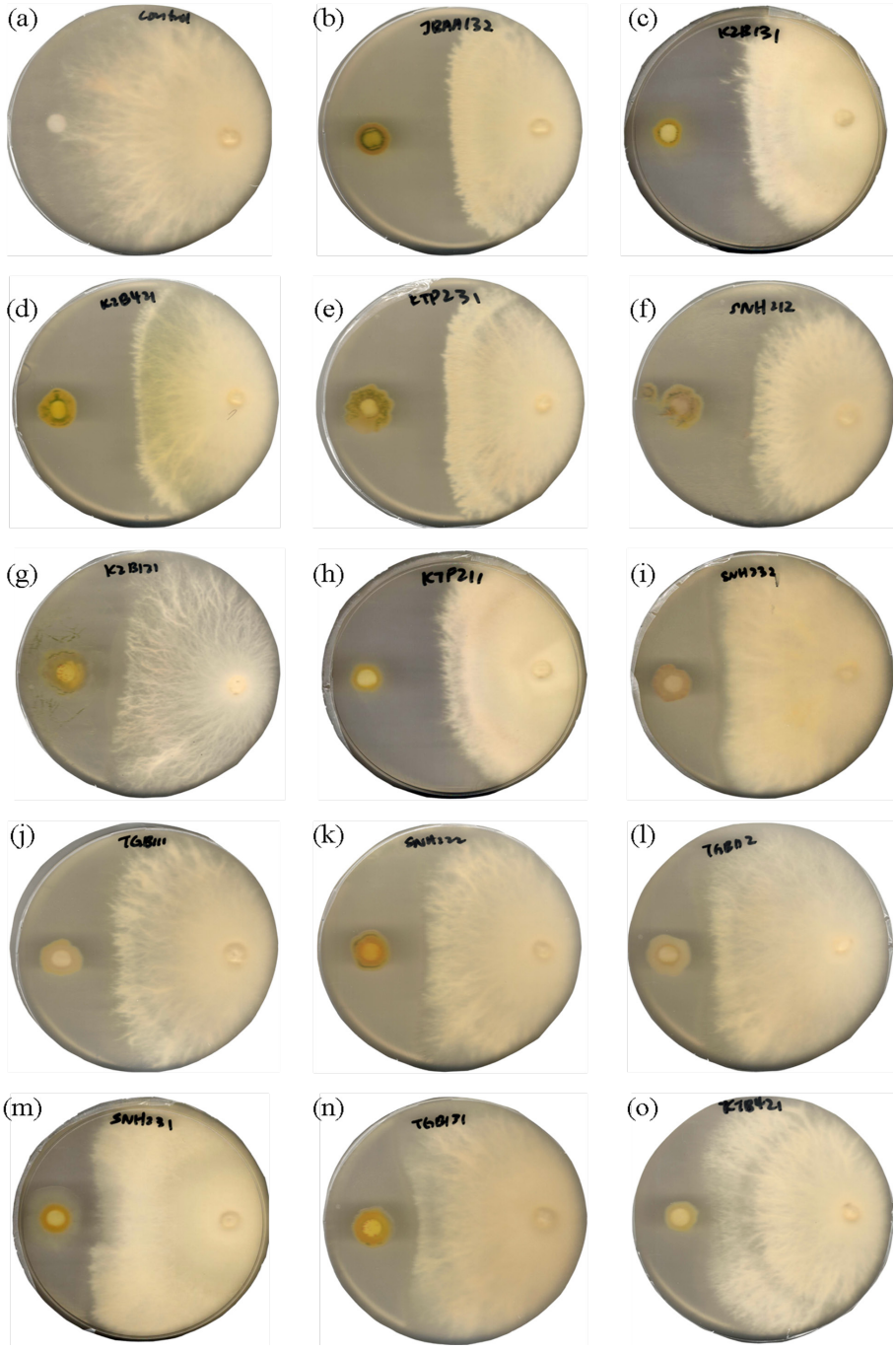

Figure 2. *In vitro* antagonistic assays. (a) Control with filter paper placed opposite a mycelial plug of Foc TR4; (b) JBAA132; (c) K2B121; (d) K2B131; (e) K2B421; (f) K2B431; (g) KTP211; (h) KTP231; (i) SNH212; (j) SNH222; (k) SNH231; (l) SNH232; (m) TGB111; (n) TGB112; (o) TGB131

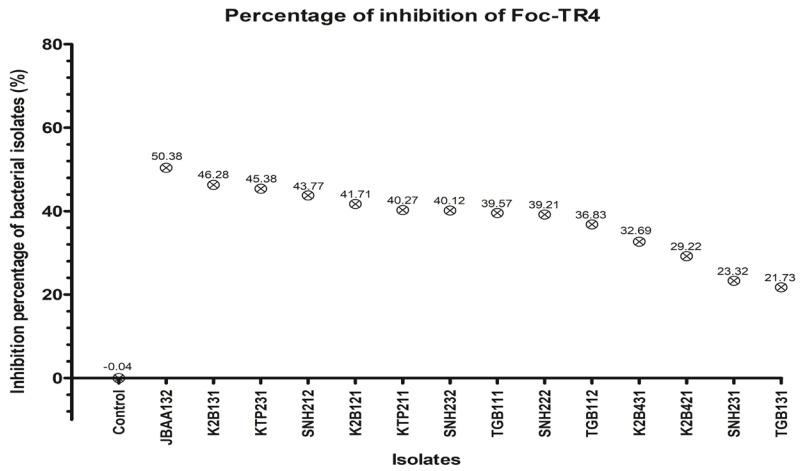

Figure 3. Percentages of inhibition of Foc TR4 by the 14 bacterial isolates

87.3% sequence similarity. The results of molecular identification via 16s rRNA based on the two databases are displayed in Table 2 and Table 3.

Ten out of 14 isolates were identified as *P. aeruginosa*, and all matched significantly to type strain DSM50071. While two isolates were identified as *Pseudomonas plecoglossicida* and *Pseudomonas taiwanensis*. The abundance of *Pseudomonas* spp. found in this study collaborated well the findings of Zhou et al. (2019), who reported that *Pseudomonas* spp. were the third most abundant genus in samples taken from banana rhizospheres. Zhou et al. (2019) also indicated that the top two dominant genera were *Bacillus* and *Lactococcus*. However, members of these genera would have likely been screened out by the siderophore production assay in our study. In another study, Kaushal et al. (2020) reported that *Pseudomonas* spp. was the second most abundant genus in Kilimanjaro revealed by an analysis of soil microbial species around infected and non-infected banana plants. Members from the genera *Bacillus*, *Paenibacillus*, *Pseudomonas*, and *Variovorax* are often associated with the plant-growth-promoting property. Thus, they are often found in close proximity with numerous plant species (Habibi et al., 2019; Saxena et al., 2020).

In addition to *Pseudomonas* spp., two non-*Pseudomonas* siderophore-producing bacteria were also present among the isolates, namely *Pantoea septica* (isolate KTP211) and *Serratia nematodiphila* (isolate SNH231). While there have been no reports of *Pantoea septica* being isolated from the soil surrounding banana plants prior to this study, Walterson and Stavrinides (2015) asserted that some members of the genus *Pantoea* isolated from soil environments have plant-growth-promoting properties. In another study, Chakdar et al. (2018) discovered a phosphate-solubilising

soil isolate similar to *Pantoea septica* LMG 5345 from termitorial soil in India, indicating its plant growth-promoting capabilities. Finally, Marcano et al. (2016) studied the bacteria community from the soil surrounding banana plants. Most of the isolates in the study came from the genus *Pseudomonas* and *Actinobacter*. However, they also identified a bacterial isolate that showed a high similarity to *Serratia nematodiphila* based on 16s rRNA sequencing.

Table 2

Percentages of similarity and coverage of antagonistic isolates capable of inhibition of Foc TR4 based on BLAST search against NCBI database

Isolate	BLAST NCBI	Similarity (%)	Coverage (%)
JBAA132	*Pseudomonas aeruginosa* DSM50071	99.48	99
K2B121	*Pseudomonas aeruginosa* DSM50071	96.68	93
K2B131	*Pseudomonas aeruginosa* DSM50071	96.51	93
K2B421	*Pseudomonas aeruginosa* DSM50071	100	100
K2B431	*Pseudomonas aeruginosa* DSM50071	97.24	91
KTP211	*Pantoea septica* LMG 5345	99.55	100
KTP231	*Pseudomonas plecoglossicida* NBRC 103162	100	100
SNH212	*Pseudomonas aeruginosa* DSM50071	99.64	100
SNH222	*Pseudomonas aeruginosa* DSM50071	99.82	100
SNH231	*Serratia nematodiphila* DZ0503SBS1	99.56	100
SNH232	*Pseudomonas aeruginosa* DSM50071	99.91	100
TGB111	*Pseudomonas taiwanensis* BCRC 17751	98.35	93
TGB112	*Pseudomonas aeruginosa* DSM50071	96.84	89
TGB131	*Pseudomonas aeruginosa* DSM50071	99.91	100

Table 3

Percentage of similarity of antagonistic isolates capable of inhibition of Foc TR4 based on similarity search against the database of Ribosomal Database Project (RDP)

Isolate	RDP	Similarity (%)
JBAA132	*Pseudomonas aeruginosa* DSM50071	87.3
K2B121	*Pseudomonas aeruginosa* DSM50071	96.7
K2B131	*Pseudomonas aeruginosa* DSM50071	80.5

Table 3 (*Continue*)

Isolate	RDP	Similarity (%)
K2B421	*Pseudomonas aeruginosa* DSM50071	99.6
K2B431	*Pseudomonas aeruginosa* DSM50071	87.1
KTP211	*Pantoea septica* LMG 5345	97
KTP231	*Pseudomonas plecoglossicida* FPC951	99.8
SNH212	*Pseudomonas aeruginosa* DSM50071	97.7
SNH222	*Pseudomonas aeruginosa* DSM50071	98.3
SNH231	*Serratia nematodiphila* DZ0503SBS1	98
SNH232	*Pseudomonas aeruginosa* DSM50071	99.3
TGB111	*Pseudomonas taiwanensis* BCRC 17751	85.2
TGB112	*Pseudomonas aeruginosa* DSM50071	96.7
TGB131	*Pseudomonas aeruginosa* DSM50071	99

Phylogenetic Analysis

The phylogenetic tree inferred from the 16S rRNA sequences showed clustering of isolates and species (Figure 4) congruent with the similarity search results. Two main clades were formed, where one of the clades (I) consisted of only *Pseudomonas* spp. In contrast, the other clade comprised only non-*Pseudomonas* species (II). The ten isolates (i.e., JBAA132, K2B121, K2B131, K2B421, K2B431, SNH212, SNH222, SNH232, TGB112, and TGB131) identified as *P. aeruginosa* based on BLAST similarity searches were grouped with various *Pseudomonas* spp. into a clade with a strong bootstrap value indicating genetic closeness. While isolate KTP231 identified as *P. plecoglossicida* and TGB111 identified as *P. taiwanensis* based on similarity searches, were clustered in the same subclade with several other *Pseudomonas* spp., which also included *P. plecoglossicida* and *P. taiwanensis*, with a strong bootstrap value of 98%. Meanwhile, isolate KTP211 was placed in the same subclade with *Pantoea septica*. In contrast, isolate SNH231, identified as *Serratia nematodiphila*, was clustered together with *Serratia marcescens* and *Serratia nematodiphila* with a strong bootstrap value of 100%.

Among the 14 fluorescent siderophore-producing isolates capable of inhibiting Foc TR4, 12 were identified as *Pseudomonas* spp. i.e., *P. aeruginosa*, *P. plecoglossicida*, and *P. taiwanensis*. Isolate JBAA132, the only isolate that displayed an *in vitro* inhibitive activity of more than 50% against Foc TR4 in this study, was identified as *P. aeruginosa*. *Pseudomonas* spp. are ubiquitous (Wu et al., 2015) and have been isolated from many environmental samples such as soil (AL-Saleh & Akbar, 2015) and water (Nasreen et al., 2015). *Pseudomonas aeruginosa* is a common soil bacterium

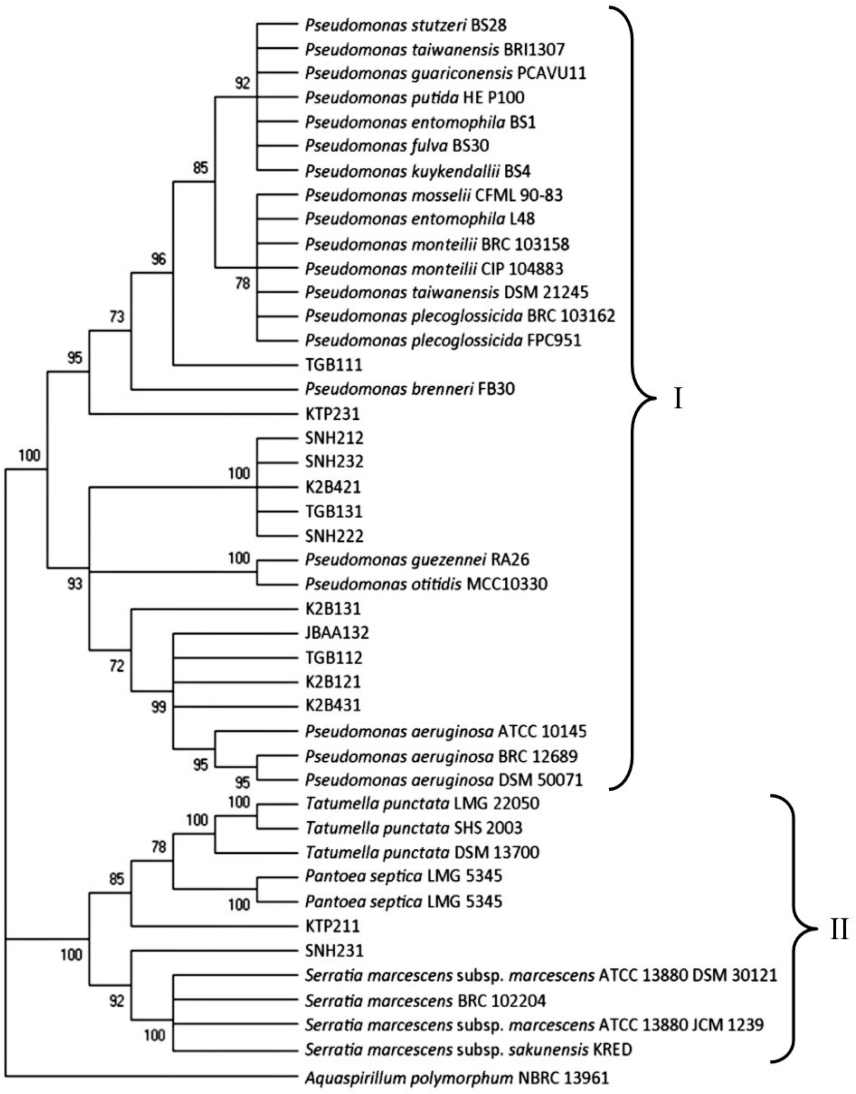

Figure 4. UPGMA tree constructed with MEGA-X using the Tamura-Nei model with 1,500 bootstraps and rooted using *Aquaspirillum polymorphum* NRBC 13961

(Gupta et al., 1999) that has been studied extensively and was reported to have biocontrol properties. Sekhar and Thomas (2015) discovered that *P. aeruginosa* was one of the bacteria capable of antagonistic ability against Foc. In addition, Lahkar et al. (2015) revealed the ability of the strain JS29 to inhibit the growth of *Alternaria solani*, the causal agent of tomato blight, by 73%. Meanwhile, *P. aeruginosa* ID 4365 and SBC 5 were demonstrated to have good antifungal ability against *Sclerotium rolfsii*,

the causal agent of Southern blight on vegetables and melons (Rane et al., 2008).

Other species within the same genus, isolate KTP231 (identified as *P. plecoglossicida*) and isolate TGB111 (identified as *P. taiwanensis*), showed moderate inhibitive activity (39.57%–45.38%) against the growth of Foc TR4 in this study. *Pseudomonas plecoglossicida* was first isolated from cultured ayu or sweetfish (*Plecoglossus altivelis*) by Nishimori et al. (2000). It was identified as a potential bioremediation agent for hazardous compounds (Boricha & Fulekar, 2009) but has not been assessed for biocontrol ability prior to this. *Pseudomonas taiwanensis* is a *Pseudomonas* species recently isolated from soil (L.-T. Wang et al., 2010). Previous studies revealed that *P. taiwanensis* is a potential biocontrol agent against plant pathogens such as *Xanthomonas axonopodis* pv. *dieffenbachiae* (Dhanya et al., 2020), and *Xanthomonas oryzae* pv. *oryzae* (Chen et al., 2016). However, no study has investigated the antagonistic activity of *P. taiwanensis* against Foc TR4.

Among the inhibitive isolates, *P. septica* and *S. nematodiphila* were the only two non-*Pseudomonas* spp. identified. Thus far, *P. septica* has never been investigated for or associated with biocontrol properties. Interestingly, isolate KTP211 (identified as *P. septica*) exhibited antagonistic activity against Foc TR4 with a moderate *in vitro* inhibition rate of 40.27% in this study. On the contrary, based on the low inhibition activity (23.32%) observed in isolate SNH231 (*S. nematodiphila*), it is unlikely a potential biocontrol agent against Foc TR4. Nonetheless, Khoa et al. (2016) had demonstrated the biocontrol properties of this species against other plant diseases such as the bacterial leaf blight in rice.

Biochemical Characterisation

Biochemical assays showed that isolate JBAA132 was catalase-positive and oxidase-positive. It demonstrated the ability to utilise citrate and mannitol and was also positive for gelatinase, tryptophanase, and cysteine desulfurase. The isolate also produced a bright green colour typical of the genus *Pseudomonas* when cultured on cetrimide agar. Isolate JBAA132 could fix nitrogen and solubilise phosphorus but could not decarboxylate arginine, asparagine, and glutamine. The results are summarised in Table 4.

There are evident differences in the biochemical characteristics between isolate JBAA132 and the type strain *P. aeruginosa* DSM50071 (Reimer et al., 2019). Type strain DSM50071 is purported to produce arginine, asparagine, and glutamine decarboxylases (Reimer et al., 2019). However, isolate JBAA132 did not produce these three types of decarboxylases. In the oxidative-fermentation test, isolate JBAA132 showed different characteristics from type strain DSM50071. Isolate JBAA132 produced a colour change in the media from green to blue when cultured in lactose and sucrose. The colour change from green to blue in the basal media indicated that even though the bacterial strain could not utilise the substrates, it could break

down the peptones contained in the basal media into alkaline products that turned the media blue (Hanson, 2008). Furthermore, isolate JBAA132 utilised dextrose under fermentative and mannitol under oxidative conditions, which were not associated with the type strain DSM50071 (Reimer et al., 2019). In addition, isolate JBAA132 was also positive for hydrogen sulphide production, which has not been reported in the type strain. It indicates a likelihood of cysteine desulfurase biosynthesis in isolating JBAA132 (J. Wang et al., 2019). Differences in the biochemical capabilities signify that isolating JBAA132 may be differ from the type strain DSM50071. This finding is congruent with the similarity results obtained from the search against the RDP, which showed only 87.3% similarity between isolate JBAA132 and the type strain. In addition, isolate JBAA132 is also capable of phosphorus solubilisation and nitrogen fixation, mechanisms found in most plant-growth-promoting bacteria (Gamalero & Glick, 2011).

Table 4

Biochemical characterisation of isolate JBAA132 and comparison with available data for Pseudomonas aeruginosa *DSM50071*

Biochemical Test		JBAA132	DSM50071
Catalase production		+	+
Cetrimide		+	+
Citrate agar		+	+
Decarboxylase activity	Arginine	-	+
	Asparagine	-	+
	Glutamine	-	+
	Lysine	-	-
	Tyrosine	-	n.a.
Gelatine liquefaction		$++_a$	+
Indole production		-	-
Lipase		-	n.a.
Methyl Red-Voges Prokaur	MR	-	-
	VP	-	-
Nitrate reduction		+	+
Nitrogen fixation		+	n.a.
Oxidase production		+	+

Table 4 (*Continue*)

Biochemical Test	JBAA132	DSM50071
Dextrose Oxidation	±$_b$	+
Dextrose Fermentation	+	-
Inulin Oxidation	±$_b$	n.a.
Inulin Fermentation	-	n.a.
Lactose Oxidation	±$_b$	-
Lactose Fermentation	-	n.a.
Sucrose Oxidation	±$_b$	-
Sucrose Fermentation	-	n.a.
Mannitol Oxidation	+	-
Mannitol Fermentation	-	n.a.
Phosphate solubilisation	+	n.a.
Motility	+	+
Hydrogen Sulphide Production	+	-

Note. $_a$ indicates very rapid liquefaction of gelatine, whereas $_b$ indicates the production of alkaline products. n.a. indicates that no information regarding this test is available at present

CONCLUSION

Antagonistic bacteria hold great potential as biocontrol agents in suppressing Foc TR4. In this study, siderophore-producing fluorescent soil isolates of genera *Pseudomonas*, *Pantoea*, and *Serratia* exhibited inhibitive activities against Foc TR4 during *in vitro* antagonistic assays were isolated. Isolate JBAA132 displayed high inhibition activity against Foc TR4 at a rate of 50.38%. Furthermore, the isolate showed high sequence similarity with *P. aeruginosa* DSM50071 based on 16s rRNA sequence searched against the NCBI database. However, it only showed 87.3% of sequence similarity to *P. aeruginosa* DSM50071 when searched against RDP. Furthermore, the isolate also displayed several biochemical characteristics different from type strain DSM50071. These findings indicate that isolating JBAA132 may be a different strain of *P. aeruginosa*. In addition, isolate JBAA132 showed biochemical activities that suggest its potential as a plant growth promoter. Therefore, it is proposed that isolates with more than 40.0% *in vitro* inhibition against Foc TR4 may be further explored for their potential *in vivo* inhibitive abilities. Nonetheless, the pathogenicity of the isolates on the animal must be investigated and determined prior to further *in vivo* studies.

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