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Potential Application of Rhizobacteria Isolated from the Central Highland of Vietnam as an Effective Biocontrol Agent of Robusta Coffee Nematodes and as a Bio-Fertilizer

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Abstract: Robusta coffee is a major commercial crop in the Central Highland of Vietnam with high economic and export value. However, this crop is adversely affected by various pathogens, particularly nematodes. This study aimed to screen active anti-nematode rhizobacterial strains for sustainable coffee production. Among more than 200 isolates, the isolate TUN03 demonstrated efficient biocontrol with nearly 100% mortality of *J2* coffee nematodes *Meloidogyne* spp. and 84% inhibition of nematode egg hatching. This active strain was identified as *Pseudomonas aeruginosa* TUN03 based on its 16S rRNA gene sequence and phylogenetic analysis. In greenhouse tests, the strain TUN03 significantly reduced the coffee nematode population in the rhizome-soil with an 83.23% inhibition rate and showed plant growth-promoting effects. Notably, this is the first report of the nematicidal effect of *P. aeruginosa* against coffee nematodes. This potent strain further showed an antifungal effect against various crop-pathogenic fungi and was found to be the most effective against *Fusarium solani* F04 (isolated from coffee roots) with a 70.51% inhibition rate. In addition, high-performance liquid chromatography analysis revealed that this bacterial strain also secretes plant growth regulators including indole acetic acid (IAA), gibberellic acid (GA3), kinetin, and zeatin in significant amounts of 100, 2700, 37, and 9.5 µg/mL, respectively. The data from this study suggest that *P. aeruginosa* TUN03 may be a potential biocontrol agent and biofertilizer for the sustainable production of Robusta coffee and other crops.

Keywords: coffee production; biocontrol; nematodes; bio-fertilizer; rhizobacteria

1. Introduction

Coffee is one of the most popular consumed beverages worldwide due to its good taste and numerous health benefits [1]. It is an important industrial crop in many countries, including Brazil, Vietnam, Colombia, Indonesia, Ethiopia, etc. Among these, Vietnam is ranked as the largest coffee producer in Asia, and the 2nd in the world for producing two major coffee species, Robusta and Arabica. Vietnam is the largest producer of Robusta coffee (May 2021 statistics of International Coffee Organization), accounting for 85% of the total production [2], which is mainly cultivated in the Central Highlands.

Coffee crops are seriously affected by plant-parasitic nematodes worldwide; in Vietnam, root-knot nematode *Meloidogyne* spp. and the lesion nematode *Pratylenchus* spp. are

the major harmful nematodes attacking coffee plantations [2–6]. More than 80% of coffee trees may suffer and eventually die due to nematode-induced diseases during the process of replanting [7]. Plants infected by nematodes show yellow leaves, stunted growth, rot root or galling, and the tree may eventually die [8–11].

Since the 1950s, chemical nematicides have been used for effective control of nematodes; however, these treatments result in environmental pollution, and affect the quality of products [12,13]. Thus, various alternative methods have been used including cultivation in a semi-arid environment along with the use of chemicals [14], co-cultivation of plants, using resistant planting material and removing infected plants [15], organic soil amendments [16], and application of herbal extracts [17] and beneficial microorganisms [18]. Among these, the use of microbes has gained high attention due to their plant-friendly and cost-effective aspects [19–21]. Microorganisms demonstrate anti-nematode activities by producing enzymes (chitinase, protease), volatile compounds, and various other secondary metabolites [11,13,22–25]. Beneficial rhizobacterial strains have been used due to their potential multiple bioactivities against pathogens and also due to the production of plant growth-promoting substances [13,26,27].

While several studies have investigated the effect of coffee nematodes in Vietnam [2–4,7,14], very few have reported the use of beneficial microbes for cost-effective biocontrol of coffee nematodes [6]. In this study, we report the isolation, selection, and identification of anti-nematode rhizobacteria in Vietnamese Robusta coffee plant roots and the assessment of their potential use in biocontrol and plant promotion effect on coffee seedlings. The plant growth-promoting compounds were also analyzed via high-performance liquid chromatography (HPLC).

2. Materials and Methods

2.1. Isolation and Identification of Rhizobacterial Strains from Robusta Coffee Roots

For isolation of rhizobacterial strains (RBs), trypticase soy agar (TSA) medium (Sigma Aldrich) was used. TSA medium was prepared by dissolving 40 g of TSA in 1 L distilled water and its initial pH was adjusted at 7, then the medium was sterilized in an autoclave for 30 min at 121 °C.

Isolation of RBs was performed according to the method reported by Nguyen et al. (2019) [13]. In brief, the soil samples were collected from the rhizosphere of healthy coffee trees and ground. One gram of soil sample was mixed with 99 mL sterile water in a plastic centrifuge tube and shaken at 200 rpm for 12 h. This was then kept still for 1 h to settle the soil, and 0.1 mL of the suspension was spread on to TSA medium and incubated at 30 °C for 1–3 d. Single colonies appearing on the plate were isolated the sub-cultured on a fresh TSA medium several times.

All RBs were stored in 50% of glycerol at −32 °C. The active bacterial strain was identified by sequencing its 16S rRNA gene according to the protocol described by Tran et al. 2018 [28]. In brief, a nearly full-length segment of the 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the universal primers 27f (5′-AGAGTTTGATCMTGGCT CAG-3′) and 1492r (5′-TACGGYTACCTTGTTACGACTT-3′). The 16S rRNA gene was amplified on an iCycler thermal cycler (Bio-Rad, USA) using the following program: 94 °C for 5 min, then 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 2 min. The amplified products were then separated by agarose gel (1.5%, *w*/*v*) electrophoresis. The target bands in the agarose gel were sliced out and purified using a QIAquick PCR purification (Promega Co., Madison, WI, USA). Sequencing reactions were carried out in a CEQ8000 Genetic Analysis System (Beckman Coulter Inc., Miami, FL, USA) using a CEQ Dye Terminator Cycle Sequencing Kit (Beckman Coulter Inc., Miami, FL, USA).

2.2. In Vitro Assay of Anti-Nematode Activity

Preparation of Bacterial Strains: The RBs were grown in trypticase soy broth (TSB, Sigma Aldrich). TSB was prepared by dissolving 20 g of TSB in 1 L distilled water, with

an initial pH of 7, then the medium was sterilized in an autoclave for 30 min at 121 °C. RBs were cultured on the TSB medium with shaking speed (150 rpm) at 28 °C for 48 h. The culture broth was centrifuged at 8000 rpm for 10 min and then kept still to settle the bacterial biomass. The bacterial mass was suspended in saline buffer and adjusted to the density of 10^7 CFU mL^{-1} after spectrophotometric measurement at 600 nm. These bacterial suspensions were used for in vitro and greenhouse tests.

Preparation of Eggs and Root-Knot Nematodes *(J2)*: *J2* Nematodes were prepared per the assay presented by Khan et al. 2008 [29]. The coffee roots were collected and washed by water to remove soil, and the egg masses collected from the roots using forceps were washed with sterile water and then with 0.5% sodium hypochlorite. The eggs were finally agitated and rinsed with sterile water on a sieve (26 µm pores). The second-stage juveniles (*J2*) were obtained by incubating the eggs for 3–5 days [30]. These nematodes *J2* were used for all subsequent tests. The process is summarized in Figure 1.

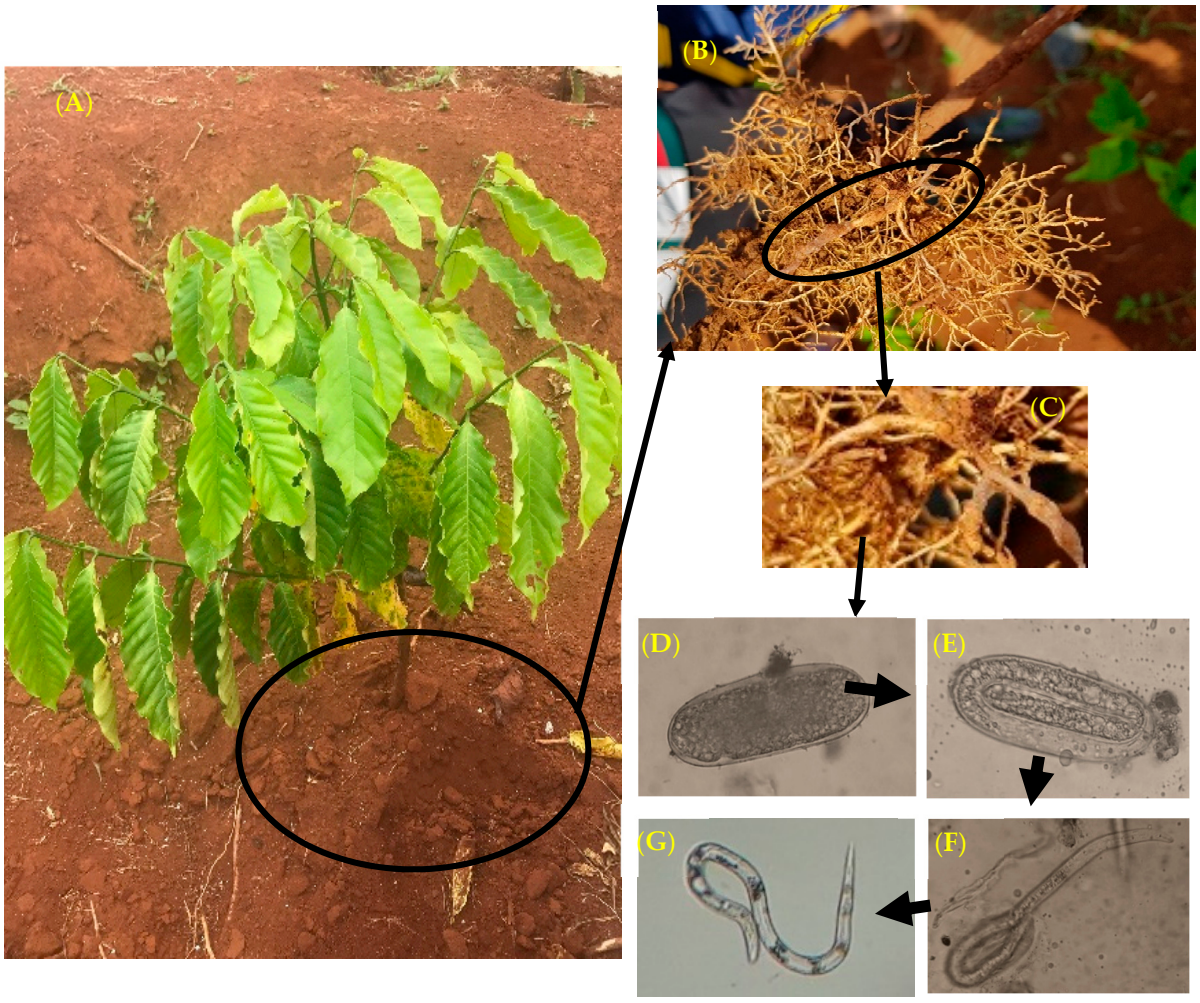

Figure 1. Preparation of eggs and root-knot nematodes (*J2*). The sick Robusta coffee (**A**) showing symptoms of nematode infection with yellow leaves was used, and its root system was harvested (**B**). Sick roots having root-knots (**C**) were collected to isolate nematode eggs (**D**), then these eggs were incubated for 3–5 days for their development (**E**) and hatching (**F**) to obtain fresh *J2* nematodes (**G**). The images of coffee nematode eggs and *J2* nematodes were observed and recorded using an Optical Olympus Microscope (Model CH30RF200, Olympus Co., Tokyo, Japan).

Anti-Nematicidal Assay: A hundred microliters of bacterial solution (10^7 CFU mL^{-1}) was mixed with 250 µL sterile distilled water containing 30 freshly hatched *J2* nematodes. The experiment was performed in a 96-well tissue culture plate at room temperature (around 28 °C), and the dead and live nematodes were counted after 24 h of incubation.

The nematodes that did not move on being touched with a fine needle were defined as dead according to the method of Cayrol et al. 1989 [31]. The dead and live nematodes were observed and are illustrated in Figure 2A,B. All the tests were performed in triplicate.

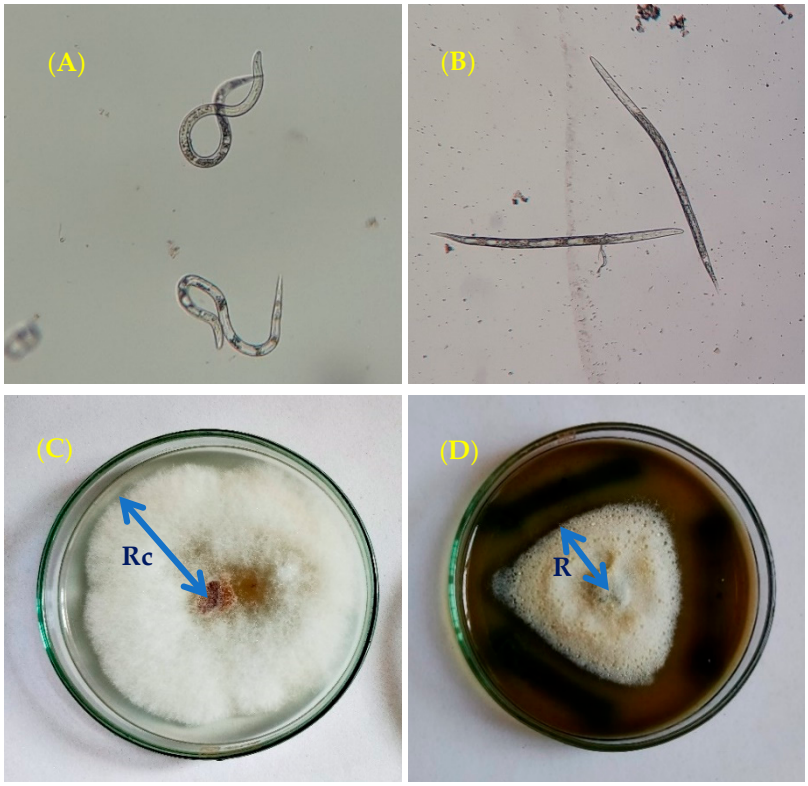

Figure 2. Images of the anti-nematicidal and anti-fungal assays of the rhizobacterial strains. The mobilized *J2* coffee nematodes (**A**) became immobilized *J2* coffee nematodes (**B**) after 24 h of treatment with rhizobacterial strain. The pathogenic fungus was grown on potato dextrose agar (PDA) (**C**), control dish, and also on PDA treated with a rhizobacterial strain (**D**), experimental dish. After five days of cultivation, the radius (cm) of fungal mycelial spread on the control dish (Rc) and the experimental dish (R) were measured for the calculation of antifungal activity.

Assay for the inhibition of nematode egg hatching: A hundred microliters of bacterial solution (10^7 CFU mL^{-1}) was mixed with 250 µL sterile distilled water containing 200 nematode eggs, and then added to a 96-well tissue culture plate. The mixture was kept at room temperature (around 28 °C) for 3 d before the hatched juveniles were counted under a low-power stereoscopic microscope [13]. All the tests were performed in triplicate.

2.3. The Effect of Rhizobacterial Strains as Anti-Nematodes and the Plant-Promoting Effect for Robusta Trees in Green Houses

Robusta coffee (TR4) seedlings were supplied by the Division of Agro-Forestry System, Western Highlands Agriculture and Forestry Scientific Institute, Buon Ma Thuot 630,000, Vietnam. The initial TR4 seedling sizes, including shoot length (17.41 cm), root length (8.43 cm), shoot dry weight (8.32 g/10 trees), and root dry weight (9.21 g/10 trees), were determined.

The effect of the selected rhizobacterial strain *Pseudomonas aeruginosa* TUN03 was further evaluated in the greenhouse test. Local Robusta coffee (TR4) seedlings with five leaves and with equal height were used for the tests. The coffee seedlings were randomly categorized into five experimental groups and in triplicate with 30 coffee seedlings per group, including two control groups: the negative control group (Group 1), and the positive control group (Group 2), as well as three experimental groups: seedlings infected with nematodes and treated with 100 mL of *Pseudomonas aeruginosa* TUN03 solution at

different densities of 0.5×10^7 CFU mL^{-1} (Group 3), 1.0×10^7 CFU mL^{-1} (Group 4), and 2.0×10^7 CFU mL^{-1} (Group 5). In the negative control group (Group 1), the coffee seedlings were not treated with bacteria and not infected with nematodes, while in the positive control group (Group 2), the seedlings were infected with nematodes without treatment of bacteria.

One coffee seedling was cultivated per pot (prepared according to the previous reports [11,32]) containing 0.6 kg of the mixture of sterilized sand, soil, and organic fertilizer in a ratio of 1:2:1 (v/v), respectively. The distance between each group and each pot were set at 60 cm and 50 cm, respectively. All the experiments were performed in a greenhouse with 75–80% humidity, temperatures between 25–30 °C, and a light intensity of 50–550 µmol m^2/s^{-1} (measured from 08 a.m. to 4 p.m.).

Three hundred *J2* nematodes were added to each pot of all groups except *Group 1*. The experimental groups were treated two times with 100 mL of *Pseudomonas aeruginosa* TUN03 solution each time on days 10 and 30 after nematode infection. Tap water was used for irrigation. The total period of this experiment was three months. The coffee seedlings were removed from the soil and washed. One gram of roots and 10 g soil were used for counting the number of J2 nematodes for calculation of the antinematode activity (%). Some growth parameters including increased shoot, root length, and dry weight (1), and the contents of some photosynthetic pigments, including chlorophyll a, chlorophyll b, and carotenoid contents (2) were also were examined.

(1) Increased shoot, root length, and dry weight were determined by comparing the shoot, root length, and dry weight of the coffee tree after three months (B) to the initial seedling size (A) according to the equation: *Increased parameters* = $B - A$.

(2) The photosynthetic pigments were examined as per the method reported by Nguyen et al. (2011) [32]. The leaves of coffee seedlings (0.5 g) were collected and cut into small pieces and immersed in 100 mL of 80% acetone for 72 h to extract the photosynthetic pigments. The extraction solvent was diluted three times and measured with a spectrophotometer at 663, 645, and 440.5 nm. The content of photosynthetic pigments was calculated using the following equations:

$$\text{Ca (mg/g fresh weight)} = (0.0127 \times OD_{663} - 0.00269 \times OD_{645}) \times 100$$

$$\text{Cb (mg/g fresh weight)} = (0.0299 \times OD_{645} - 0.00468 \times OD_{663}) \times 100$$

$$\text{Ccar (mg/g fresh weight)} = (0.004695 \times OD_{440.5} - 0.000268\ (\text{Ca} + \text{Cb})) \times 100$$

where Ca, Cb (mg g^{-1} fresh leaf) are content of chlorophyll a and b; and Ccar (mg/g fresh weight) is carotenoid content.

2.4. Anti-Fungal Assays

Anti-fungal activity of *Pseudomonas aeruginosa* TUN03 was assessed according to the method per Ngo et al. 2020 [33]. A total of 12 fungal pathogen strains (supported from the Institute of Biotechnology and Environment, Tay Nguyen University, Buon Ma Thuot, Vietnam), including *Purpureocillium lilacinum* F01, *Fusarium solani* F02, *F. solani* F03, *F. solani* F04, *Colletotrichum gloeosporioides* F05, *F. incarnatum* F06, *Gongronella butleri* F07, *Pestalotiopsis mangiferae* F08, *F. incarnatum* F09, *F. oxysporum* F10, *Neonectria* sp. F11, and *F. incarnatum* F15. A mycelial plug of growing fungal strain was placed in the center of the Petri dish containing PDA (potato D-glucose agar) medium, and three lines of *Pseudomonas aeruginosa* TUN03 were streaked at a distance of 2 cm from the center of the surface of PDA medium in Petri dishes (Figure 1). The experiment was maintained at 28 °C for 5 d. The radial growth of fungal mycelium was measured, and the anti-fungal activity was calculated according to the equation:

$$\text{Anti-fungal activity (\%)} = [1 - R/Rc] * 100\%$$

The radius (cm) of fungal mycelium cultivated on the control dish (not treated with TNU3 strain) and experimental dish (treated with TNU3 strain) are presented as Rc and R, respectively (Figure 1C,D). All the tests were done with triplicate.

2.5. High-Performance Liquid Chromatography (HPLC) Analysis of Plant-Promoting Compounds

King B media (Sigma Aldrich) was used for cultivation of *Pseudomonas aeruginosa* TUN03 and was prepared in distilled water. Twenty grams of King B media powder was dissolved in 1 L distilled water and its initial pH was adjusted to 7, then the medium was sterilized in an autoclave for 30 min at 121 °C.

The contents of plant growth-promoting compounds (PGPCs), including IAA, GA3, kinetin, and zeatin were determined by using the high-performance liquid chromatography (HPLC; UHPLC-UV Ultimate 3000, Thermo, Germering, Germany) technique. The residues and bacterial biomass in culture broths of *Pseudomonas aeruginosa* TUN03 were removed by centrifugation at 8000 rpm in 10 min and the harvested culture supernatants were used to detect and determine the concentration of some PGPCs. Five microliters of culture supernatants were injected into the HPLC system and separated via a C18 column (BDS Hypersil C18 (250 × 4.6 × 5)). The PGPCs were detected at a wavelength of 254 nm under the following analysis conditions: mobile phase 60 methanol in water adjusted at pH 5.8 using 10 mM ammonium acetate with a flow rate of 0.8 mL, at a column temperature of 30 °C for 10 min. Commercially available substances, including IAA, GA3, kinetin, and zeatin (Merck KGaA, Darmstadt, Germany) with purities of 98%, 97%, 99%, and 95%, respectively, were used as standards for calculating the concentration of compounds in the culture supernatants as following equations:

$$\text{For the determination of IAA } (\mu g/mL): y = 0.0629x + 0.2191;\ R^2 = 0.990$$

$$\text{For the determination of GA3 } (\mu g/mL): y = 0.0098x + 0.0051;\ R^2 = 0.9995$$

$$\text{For the determination of Kinetin } (\mu g/mL): y = 0.3777x - 0.4242;\ R^2 = 0.998$$

$$\text{For the determination of Zeatin } (\mu g/mL): y = 0.5633x + 0.3056;\ R^2 = 0.9992$$

where x is concentration of PGPCs and y is the peak area of commercial PGPCs. In addition, all the tests were done in triplicate.

2.6. Statistical Analysis

All the experiments were of a randomized design. The experimental data on anti-nematodes, anti-fungi, and plant growth-promoting effects were obtained and analyzed via simple variance (ANOVA) followed by Duncan's multiple range test (when the experiment contains $\geq$6 items that need to be compared) and Fisher's LSD test (when the experiment contains $\leq$5 items that need to be compared) at $p = 0.05$. Statistical Analysis Software (SAS-9.4) purchased from SAS Institute Taiwan Ltd. (Taipei, Taiwan) was used for statistical analysis.

3. Results and Discussion

3.1. Isolation, Evaluation, and Identification of the Most Active Anti-Nematodes Rhizobacterium via In Vitro Tests

More than 200 rhizobacterial strains were isolated from the rhizosphere soil samples collected from Robusta coffee fields in the Central Highland of Vietnam. These isolated strains were evaluated for their anti-nematode activity. The experimental results indicated that 24 strains demonstrate a high anti-nematicidal activity, with a *J2* nematode mortality value of $\geq$60%; of these, some strains (TUN03, TUN67, and TUN85) displayed the most potent effect against *J2* nematodes, with mortality value in the range of 92.36–98.26% (Table 1). For further evaluation of the anti-nematode efficacy of these 24 strains, the coffee nematode egg hatching inhibitory assay was also performed. The data were recorded and are presented in Table 1. These isolates showed nematode egg hatching inhibition in the

range of 35.80–84.03%, and the strain TUN03 was also found to be the most effective strain, with the highest egg hatching inhibition value of 84.03%. Thus, this potential bacterium was chosen for further investigation. Based on the 16S rDNA gene sequence analysis, this bacterial strain was identified as *Pseudomonas aeruginosa*, with a similarity rate of 100%. The phylogenetic analysis of the identified strain is illustrated in Figure 3, and the 16s rRNA gene sequence of this identified strain was submitted in DDBJ/EMBL/Genbank (accession number: LC645701).

Table 1. Nematicidal activity of some anti-nematode rhizobacteria isolated in the Central Highland of Vietnam.

No	Sample ID	Nematicidal Activity (%)	
		Mortality of *J2* Nematodes	Inhibition Against Egg Hatch
1	TUN01	87.38 ± 0.72 ^{d}	66.07 ± 0.43 ^{fg}
2	TUN03	98.26 ± 0.21 ^{a}	84.03 ± 0.90 ^{a}
3	TUN07	64.92 ± 0.98 ^{kl}	77.70 ± 1.02 ^{b}
4	TUN11	71.98 ± 1.31 ^{hi}	44.95 ± 2.12 ^{j}
5	TUN22	89.06 ± 1.42 ^{cd}	36.50 ± 0.46 ^{l}
6	TUN31	69.21 ± 1.78 ^{ij}	70.04 ± 1.02 ^{de}
7	TUN45	89.68 ± 0.57 ^{cd}	69.25 ± 1.84 ^{e}
8	TUN47	66.40 ± 0.25 ^{jk}	37.44 ± 0.48 ^{kl}
9	TUN66	78.59 ± 1.25 ^{f}	65.48 ± 0.41 ^{gh}
10	TUN67	92.36 ± 1.20 ^{bc}	69.47 ± 0.72 ^{e}
11	TUN85	94.45 ± 0.69 ^{b}	72.83 ± 1.36 ^{cd}
12	TUN99	68.44 ± 2.04 ^{jk}	35.80 ± 0.92 ^{l}
13	TUN108	77.88 ± 0.44 ^{fg}	43.39 ± 0.42 ^{j}
14	TUN121	83.38 ± 1.49 ^{e}	68.23 ± 0.29 ^{fg}
15	TUN145	89.21 ± 0.54 ^{cd}	74.39 ± 1.74 ^{c}
16	TUN147	66.51 ± 0.25 ^{jk}	43.88 ± 0.68 ^{j}
17	TUN169	74.31 ± 1.24 ^{gh}	35.13 ± 1.47 ^{l}
18	TUN178	88.69 ± 1.49 ^{cd}	68.73 ± 1.25 ^{ef}
19	TUN179	65.35 ± 2.24 ^{kl}	45.73 ± 1.23 ^{j}
20	TUN184	60.01 ± 0.35 ^{m}	39.65 ± 1.15 ^{k}
21	TUN198	75.71 ± 1.31 ^{gh}	55.57 ± 0.39 ^{i}
22	TUN199	77.83 ± 1.55 ^{fg}	62.90 ± 0.88 ^{h}
23	TUN203	61.85 ± 1.88 ^{lm}	37.64 ± 0.77 ^{kl}
24	TUN205	89.20 ± 0.76 ^{cd}	71.24 ± 1.41 ^{de}
F Value		85.27	248.82
Pr > F		<0.0001	<0.0001
P		0.05	0.05
CV%		2.753726	3.061748

Rhizobacterial bacterial strains were cultivated in TSB medium with shaking speed (150 rpm) at 28 °C for 48 h. Bacteria were harvested by centrifuging the cultured medium at 8000 rpm for 10 min. The supernatant was removed by centrifugation at 8000 rpm for 10 min to settle the bacterial biomass, and then each bacterial mass was suspended in the saline buffer to adjust the density to 10^7 CFU mL^{-1} for antinematode activity assay. All the experiments were performed in triplicate. The data were analyzed via simple variance (ANOVA), and then Duncan's multiple range test at $p = 0.05$ was performed. Values in the same column with the different letters are significantly different.

Earlier reports prove that *Pseudomonas aeruginosa* has a wide range of important applications in environment management [34–36], in industry for the production of various secondary metabolites (Rhamnolipid, Vanillin, enzymes, and pigments) [36–41], as well as its potential use in agriculture [42–51]. This bacterial genus has been proven to have potential use as a plant growth promoter [42] and produces an anti-plant virus agent [43]. In particular, *Pseudomonas aeruginosa* and its secondary metabolites have been widely used for controlling numerous plant-pathogen fungal strains [44–48]. However, there are several reports on the assessment of nematicidal activity of *Pseudomonas aeruginosa* against several

nematodes, including banana, tomato, and okra root-knot nematodes [49–51]. There is also no report of the nematicidal effect of this genus against coffee nematodes. Thus, this study shows for the first time the potential nematicidal effect of *Pseudomonas aeruginosa* against coffee nematodes *J2* and its egg hatching and adds to the existing knowledge of the biological activities of *Pseudomonas aeruginosa.*

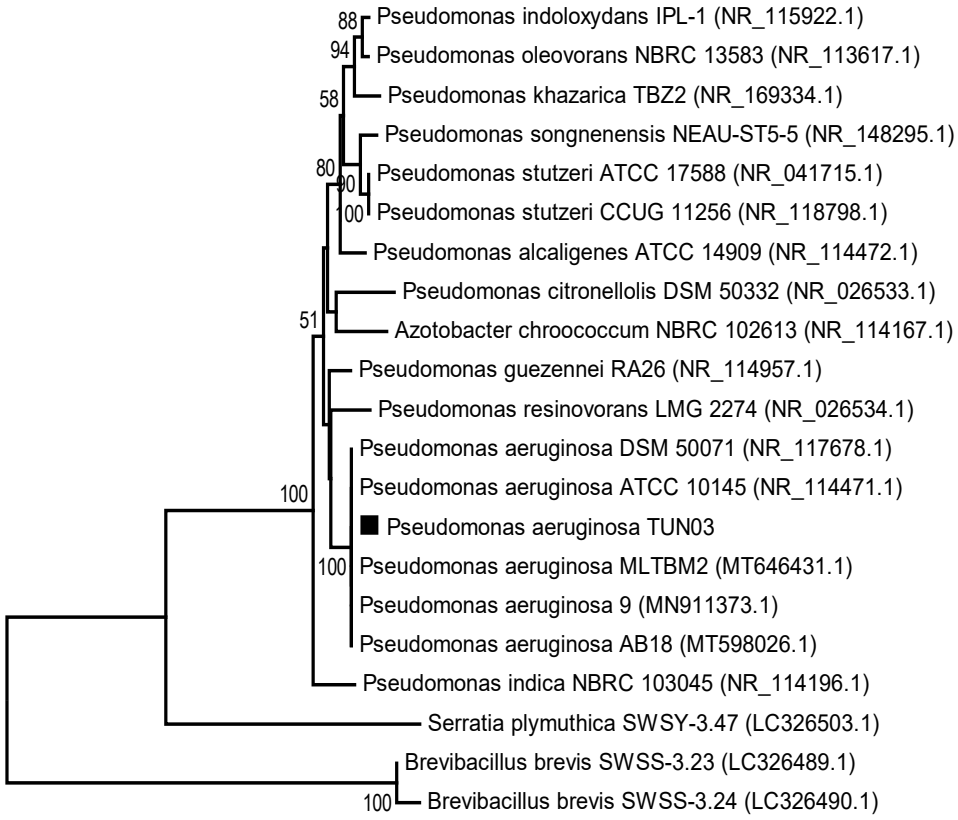

Figure 3. Phylogenetic analysis of the rhizobacterium *Pseudomonas aeruginosa* TUN03 based on the 16s rRNA gene sequences. The phylogenetic tree was constructed using Kimura's method and Mega software version 6.0 after multiple alignments of the data by ClustalW. The numbers at the branches are bootstrap confidence percentages (%).

3.2. Assessment of the Potential Biocontrol of Robusta Coffee Nematodes and Plant-Promoting Effect of Pseudomonas aeruginosa TUN03 under Greenhouse Conditions

Pseudomonas aeruginosa TUN03 was chosen for further experimental study in greenhouse tests. Robusta coffee TR4 strain is the most cultivated in the Central Highland of Vietnam; therefore, the TR4 coffee seedlings were used as a plant model to test according to the protocol presented in the Methods Section 2.3. A summary of the nematicidal effect of *Pseudomonas aeruginosa* TUN03 in the greenhouse is presented in Table 2.

After three months of infection with nematodes, the density of J2 nematodes in soils and roots of coffee seedlings in group 2 were much higher than those of group 1. This result indicated the seedlings were successfully infected with nematodes, and the experiment data are qualified enough for analysis. Furthermore, all the three experimental groups treated with *Pseudomonas aeruginosa* TUN03 showed a significant effect on reducing nematodes in soils and in roots compared to the positive control group (Group 2). The treatment bacterial density of 1.0×10^7 CFU mL^{-1} (Group 4) and 2.0×10^7 CFU mL^{-1} (Group 5) demonstrated great nematicidal effect with a high reduction of 78.8% and 83.1% of *J2* nematodes in rhizosphere soils and 42.7% and 52.1% in coffee roots, respectively. In addition, the death rate of coffee seedlings in these treated groups and the negative control group (Group 1) was low, in the range of 7.7–8.3%, while the death rate of coffee seedlings

in the positive group (Group 2) and Group 3 was found to be rather high, at 37.3% and 18.3%, respectively. Thus, treatment with all densities of *Pseudomonas aeruginosa* TUN03 did not harm the coffee seedlings in the assays. In addition, these experimental groups treated with *Pseudomonas aeruginosa* TUN03 (Groups 4 and 5) showed positive plant promoting effect on coffee seedlings (Table 3), including the dramatically increased shoot length (47.7–48.1 cm), and increased shoot dry weight (32.6–34.1 g/10 trees) compared to those in other groups. The roots of these coffee trees in Groups 4 and 5 seemed stronger and healthier (Figure 4) than those in control groups, as evidenced by measuring the root dry weight. As shown in Table 3, all the groups treated by *Pseudomonas aeruginosa* TUN03 showed a much higher root dry weight (30.2–30.4 g/10 trees) than those in the control groups (20.6–22.5 g/10 trees).

Table 2. The effect of *Pseudomonas aeruginosa* TUN03 on the density of coffee nematodes in soils and coffee roots and the death rate of coffee seedlings cultivated under greenhouse conditions.

Treatments Group	Nematodes in Rhizosphere Soils		Nematodes in Coffee Roots		DR * (%)
	Nematodes *J2* in 10 g Soil (Count)	Reduction (%)	Nematodes *J2* in 1 g Root (Count)	Reduction (%)	
Group 1 (negative control)	22.0 ± 1.53 ^{c}	-	17.7 ± 2.33 ^{c}	-	8.2 ± 1.42 ^{c}
Group 2 (positive control)	156.7 ± 8.82 ^{a}	-	48.4 ± 4.31 ^{a}	-	37.3 ± 3.71 ^{a}
Group 3	58.3 ± 4.41 ^{b}	58.3 ± 3.15	28.7 ± 2.19 ^{b}	28.9 ± 5.42	18.3 ± 1.67 ^{b}
Group 4	29.7 ± 4.84 ^{c}	78.8 ± 3.46	23.1 ± 2.63 ^{bc}	42.7 ± 6.51	8.3 ± 1.67 ^{c}
Group 5	23.7 ± 2.96 ^{c}	83.1 ± 2.12	19.3 ± 0.333 ^{c}	52.1 ± 0.83	7.7 ± 2.03 ^{c}
F Value	148.85	-	19.48	-	30.62
Pr > F	<0.0001	-	0.0003		<0.0001
P	0.05		0.05		0.05
CV%	13.94488	-	17.83826	-	24.99341
LSD	15.246	-	9.2175	-	7.5137

DR *: Death rate of coffee seedlings (%). All the experiments were performed in triplicate. The data were analyzed via simple variance (ANOVA), then Fisher's LSD test at $p = 0.05$ was performed. Values in the same column with different letters are significantly different. LSD: Least Significant Difference.

Table 3. The effect of *Pseudomonas aeruginosa* TUN03 on some plant promoting parameters of the coffee seedlings grown under greenhouse conditions.

Treatments Group	Promoting Shoots		Promoting Roots	
	Increased Length (cm)	Increased DW (g/10 Trees)	Increased Length (cm)	Increased DW (g/10 Trees)
Group 1 (negative control)	35.0 ± 2.89 ^{bc}	19.8 ± 2.89 ^{c}	16.5 ± 1.44 ^{a}	22.5 ± 1.82 ^{b}
Group 2 (positive control)	28.7 ± 2.404 ^{c}	17.6 ± 1.48 ^{c}	16.3 ± 1.88 ^{a}	20.6 ± 2.80 ^{b}
Group 3	44.3 ± 2.85 ^{ab}	28.8 ± 3.89 ^{b}	16.8 ± 1.07 ^{a}	30.2 ± 2.23 ^{a}
Group 4	47.7 ± 2.61 ^{a}	34.1 ± 2.37 ^{a}	17.1 ± 1.82 ^{a}	30.9 ± 1.44 ^{a}
Group 5	48.1 ± 3.93 ^{a}	32.6 ± 2.59 ^{a}	17.0 ± 1.73 ^{a}	30.4 ± 1.46 ^{a}
F Value	6.95	69.75	1.11	68.56
Pr > F	0.0102	<0.0001	0.4160	<0.0001
P	0.05	0.05	0.05	0.05
CV%	13.82137	5.838377	3.493248	3.865095
LSD	10.607	2.9213	1.1019	1.958

DW: dry weight of ten coffee seedlings (g/10 trees). All the experiments were performed in triplicate. The data were analyzed via simple variance (ANOVA), then Fisher's LSD test at $p = 0.05$ was performed. Values in the same column with different letters are significantly different. LSD: Least Significant Difference.

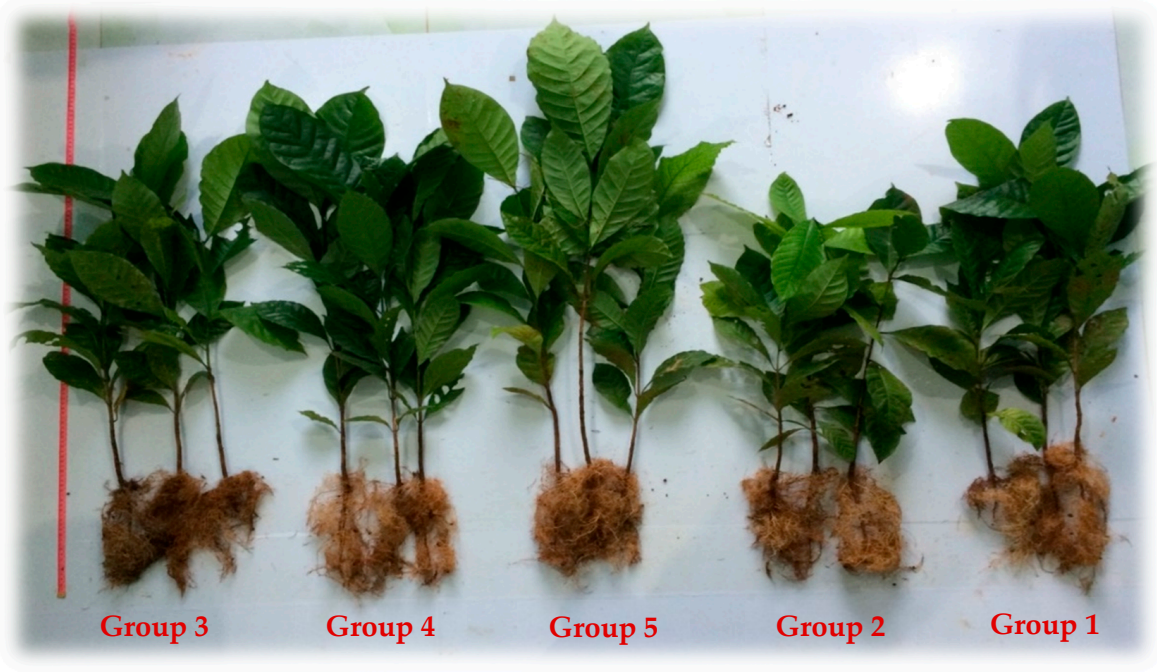

Figure 4. The harvested coffee seedlings after three months of experimental cultivation in the greenhouse. The coffee seedlings were randomly divided into two control groups in which the coffee seedlings were not treated with bacteria and not infected with nematodes (Group 1) or infected with nematodes without treatment of bacteria (Group 2), and three experimental groups: infected nematodes and treated with 100 mL of *Pseudomonas aeruginosa* TUN03 solution at different densities of 0.5×10^7 CFU mL^{-1} (Group 3), 1.0×10^7 CFU mL^{-1} (Group 4), and 2.0×10^7 CFU mL^{-1} (Group 5).

The effect of *Pseudomonas aeruginosa* TUN03 on the content of photosynthetic pigments, including chlorophyll a, chlorophyll b, and carotenoid, in the leaves of the coffee seedlings cultivated under greenhouse conditions was also observed and the results are recorded in Table 4. This bacterial strain enhanced photosynthetic pigment content in the leaves of coffee seedlings. Among the treated groups, Group 4 showed a slightly higher content of photosynthetic pigments than in Group 5 and much higher than those in other groups. Based on the experimental results, the rhizobacterium *Pseudomonas aeruginosa* TUN03 was found to be a potential candidate for biocontrol of coffee nematodes as well as a good plant growth enhancer for this crop.

Table 4. The effect of *Pseudomonas aeruginosa* TUN03 on the levels of photosynthetic pigments in the leaves of the coffee seedlings grown under greenhouse conditions.

Treatments Group	Content of Photosynthetic Pigments (mg/g Fresh Weight)		
	Chlorophyll [a]	Chlorophyll [b]	Carotenoid
Group 1 (negative control)	0.84 ± 0.087^{c}	0.51 ± 0.023^{d}	0.413 ± 0.022^{c}
Group 2 (positive control)	0.57 ± 0.073^{c}	0.35 ± 0.026^{e}	0.337 ± 0.026^{d}
Group 3	0.94 ± 0.088^{b}	0.54 ± 0.033^{c}	0.480 ± 0.012^{c}
Group 4	1.22 ± 0.093^{a}	0.73 ± 0.032^{a}	0.557 ± 0.012^{a}
Group 5	1.19 ± 0.116^{a}	0.70 ± 0.029^{b}	0.550 ± 0.029^{ab}
F Value	6.87	764.32	17.86
Pr > F	0.0106	<0.0001	0.0005
P	0.05	0.05	0.05
CV%	18.63119	1.724079	8.199466
LSD	0.3335	0.0184	0.0721

All the experiments were performed in triplicate. The data were analyzed via simple variance (ANOVA), then Fisher's LSD test at $p = 0.05$ was performed. Values in the same column with the different letters are significantly different. LSD: Least Significant Difference.

3.3. Potential Application of P. aeruginosa TUN03 as a Potent Biofertilizer Agent Related to Large-Scale Production of Plant Growth Promoting Compounds (PGPCs)

For a further understanding of the potential plant-promoting effect of *Pseudomonas aeruginosa* TUN03 on the coffee seedlings in the previous experiments (Section 3.2), we used the HPLC technique to explore its ability to synthesize PGPCs. This bacterium was cultivated in King B medium broth at 28 °C for two days. Analysis of the fermented culture broth supernatant revealed the production of some major PGPCs including GA3, IAA, zeatin, and kinetin through HPLC fingerprinting as major peaks at the retention times of 3.172, 3.218, 3.963, and 4.662 min, respectively (Figure 5). Of these, the highest peak was that of GA3, indicating that this compound was produced at the highest yield among the PGPCs in the culture broth. To clarify this result, the concentrations of these four PGPCs were determined using the equations (presented in Section 2.5) created by using the commercial PGPCs as standard compounds. The yields of these PGPCs were 231 µg/mL (IAA), 2702 µg/mL (GA3), 36.8 µg/mL (kinetin), and 7.71 µg/mL (zeatin). In addition, the culture broth contained a dark green pigment, which is well-known to be Pyocyanin, produced by *Pseudomonas aeruginosa* [46,52]. Pyocyanin is a potential biocontrol compound against a vast array of plant-pathogen diseases [53], and as such, it may be a potent plant-promoting agent via its indirect mode of action [11,13,54–57]. Pyocyanin was found to be produced at 16 µg/mL, which was determined using the method published by Devnath et al. (2017) [58]. Because of its high anti-pathogenic activity (anti-nematode effect) and biosynthesis of multi PGPCs at a high level, these data may prove *Pseudomonas aeruginosa* TUN03 to be a potential biofertilizer for use in agriculture.

Biosynthesis of IAA by *Pseudomonas aeruginosa* has been widely reported [59–68]; however, only a few studies have reported the production of GA3 by *Pseudomonas aeruginosa* [67–69]. Although cytokinin compounds have also been reported to be produced by *Pseudomonas aeruginosa*, there are quite a few reports on the biosynthesis of zeatin, and kinetin by this bacterial genus [68,70–72]. Therefore, the results obtained in this study also contribute to enriching the available data on kinetin and zeatin biosynthesis by *Pseudomonas*.

The comparison of the yield of PGPCs produced by different *Pseudomonas aeruginosa* strains in different reports is summarized in Table 5. The level of cytokinin compounds produced by *Pseudomonas aeruginosa* TUN03 (7.71–36.8 µg/mL) was comparable to those produced by earlier reported strains (1.32–30 µg/mL); however, IAA was produced by TUN03 at a much higher yield (231 µg/mL) than that reported earlier (4.9–74.54 µg/mL). Notably, GA3 was biosynthesized by *Pseudomonas aeruginosa* TUN03 at a remarkable concentration of 2702 µg/mL (based on HPLC determination), which is a new record of high biosynthesis of GA3 by *Pseudomonas aeruginosa*.

The ability to produce IAA, especially GA3, by *Pseudomonas aeruginosa* TUN03 isolated in this study was different from that of other previously reported strains, possibly due to various factors, such as variation in fermentation conditions, different carbon/nitrogen sources, and particularly, detection by different methods and types of equipment. HPLC analysis (the most widely accepted for quantity and concentration determination of various chemical compounds) was used in this study for the detection and concentration determination of these active compounds. Thus, we suggest that the bacterial strain isolated in this study possesses a significant characteristic of IAA and GA3 biosynthesis at a high level.

3.4. Novel Potential Anti-Fungal Effect of Pseudomonas aeruginosa TUN03

To further investigate the potential use of this strain in agriculture, we explored its antifungal activities against various plant-pathogen fungal strains that seriously damage some crops, such as coffee, black pepper, Durio, Persea, Dimocarpus, knotweed, citrus, and sweet potato, cultivated in the Central Highland of Vietnam. A total of 12 pathogen fungal strains were used for the tests. The data are summarized and recorded in Table 6 and also illustrated in Figure 6.

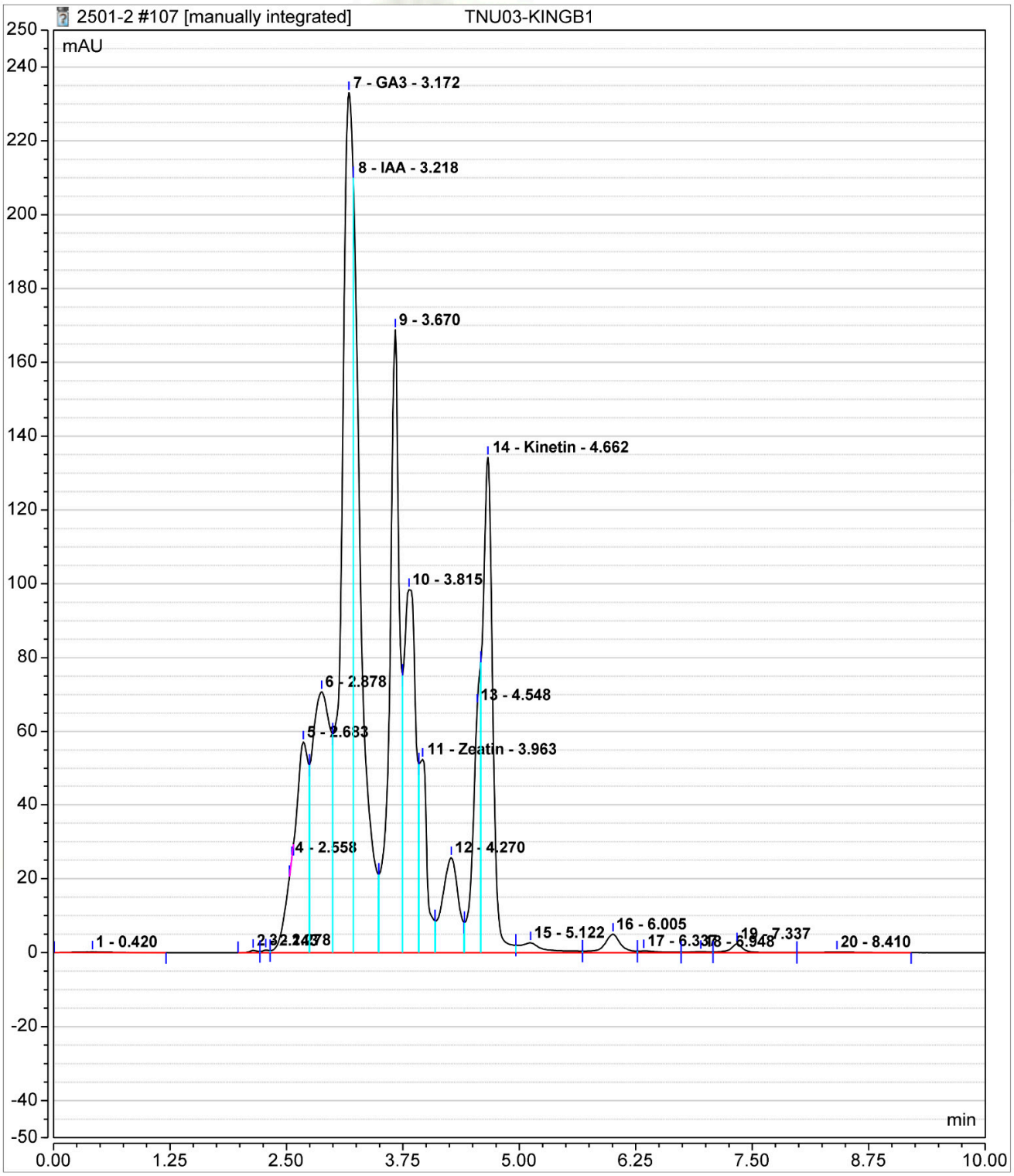

Figure 5. The HPLC profiles of production of plant-promoting compounds (PGPCs) biosynthesized by *Pseudomonas aeruginosa* TUN03. This bacterial strain was cultivated in King B medium at 28 °C for two days. The fermented culture broth was centrifugated at 8000 rpm for 10 min to remove the residues and bacterial biomass and the supernatant was collected to be used for the detection of PGPCs. Five microliters of culture supernatant were injected into the HPLC system and separated on a C18 column, the PGPCs were detected at the wavelength of 254 nm under the following analysis conditions: mobile phase 60% methanol in water adjusted to pH 5.8 using 10 mM ammonium acetate with a flow rate of 0.8 mL, and a column temperature of 30 °C for 10 min.

Table 5. A comparison of plant-promoting compounds (PGPCs) produced by *Pseudomonas aeruginosa* strains in different studies.

Strains	PGPCs	Yield (μg/mL)	Medium	References
P. aeruginosa TUN03	IAA	231	King B	This study
	GA3	2702	King B	
	Kinetin	36.8	King B	
	Zeatin	7.71	King B	
P. aeruginosa Tad-21	IAA	24.54	LB + Tryp	[59]
P. aeruginosa 2apa	IAA	4.9	King B	[60]
P. aeruginosa PUPa3	IAA	26.6	LB + Tryp	[61]
P. aeruginosa OSG41	IAA	32	LB + Tryp	[62]
P. aeruginosa NJ-15	IAA	20	NB + Tryp	[63]
P. aeruginosa	IAA	13.2	LB + Tryp	[64]
P. aeruginosa BRp3	IAA	30	LB	[65]
P. aeruginosa RTE4	IAA	74.54	Mannitol + Tryp	[66]
P. aeruginosa MAJ PIA03	IAA	27.84	PIA	[67]
P. aeruginosa ZNP1	IAA	15	PIA	[67]
P. aeruginosa ZNP1	IAA	15	LB	[68]
P. aeruginosa An-1-kul	GA3	485.8	King B	[69]
P. aeruginosa An-13-kul	GA3	419.2	King B	[69]
P. aeruginosa MAJ PIA03	GA3	8.21	PIA	[67]
P. aeruginosa PM389	GA3	7.5	LB	[68]
P. aeruginosa PS 3	Cytokinin	2.96	King B	[70]
P. aeruginosa PS 2	Cytokinin	1.32	King B	[70]
Pseudomonas sp-M	Cytokinin	30	King B	[71]
P. aeruginosa PM389	Trans-Zeatin	12	LB	[68]
P. aeruginosa UPMP3	Zeatin	TLC detection	NB + Tryp	[72]

Table 6. Assessment of in vitro antifungal activity test of *Pseudomonas aeruginosa* TUN03 against various plant-pathogen fungal strains.

No	Fungal Strains	Fungal Origin	Anti-Fungal Activity (%)
1	*P. lilacinum* F01	Dimocarpus roots	+
2	*F. solani* F02	Durio roots	41.67 ± 0.58^{d}
3	*F. solani* F03	Pepper roots	30.66 ± 0.90^{f}
4	*F. solani* F04	Coffee roots	70.51 ± 2.56^{a}
5	*C. gloeosporioides* F05	Sweet potato roots	30.00 ± 0.88^{g}
6	*F. incarnatum* F06	Citrus roots	44.15 ± 1.92^{c}
7	*G. butleri* F07	Pepper roots	50.00 ± 1.33^{b}
8	*P.mangiferae* F08	*Persea americana* roots	35.19 ± 1.06^{e}
9	*F. incarnatum* F09	*Hylocereus undatus* roots	15.09 ± 0.36^{j}
10	*F. oxysporum* F10	Pepper roots	0.00 ± 0.00^{k}
11	*Neonectria sp.* F11	Durio fruits	20.50 ± 0.53^{i}
12	*F. incarnatum* F15	Knotweed leaves	28.57 ± 0.35^{h}
	F Value		10,751.3
	Pr > F		<0.0001
	P		0.05
	CV%		0.938284

(+): the activity was observed but not clear for harvesting the data based on the method reported by Ngo et al. 2020 [33]. All the experiments were performed in triplicate. The data were analyzed via simple variance (ANOVA), then Duncan's multiple range test at $p = 0.05$ was performed. Values with the different letters are significantly different. *Pseudomonas aeruginosa* has been reported to exhibit potent antifungal activity against some pathogen fungus that seriously damage various crops, including rice, groundnut, tobacco, chili, mango, sugarcane, tea, cotton, banana, and tomato [44,45,47]. In this study, we first evaluated the antifungal activity of *Pseudomonas aeruginosa* TUN03 against the pathogen various plant-pathogen fungal strains which seriously damaged some crops (listed in Table 6) cultivated in the Central Highland of Vietnam. Interestingly, this strain displayed a high inhibitory effect against *F. solani* F04 which was isolated from the roots of sick coffee (yellow leaves) plants. This finding proved that *Pseudomonas aeruginosa* TUN03 could be a potential candidate for biocontrol and biofertilizer of coffee and other crops. This strain needs to be examined for its potential application on Robusta coffee plants under field conditions.

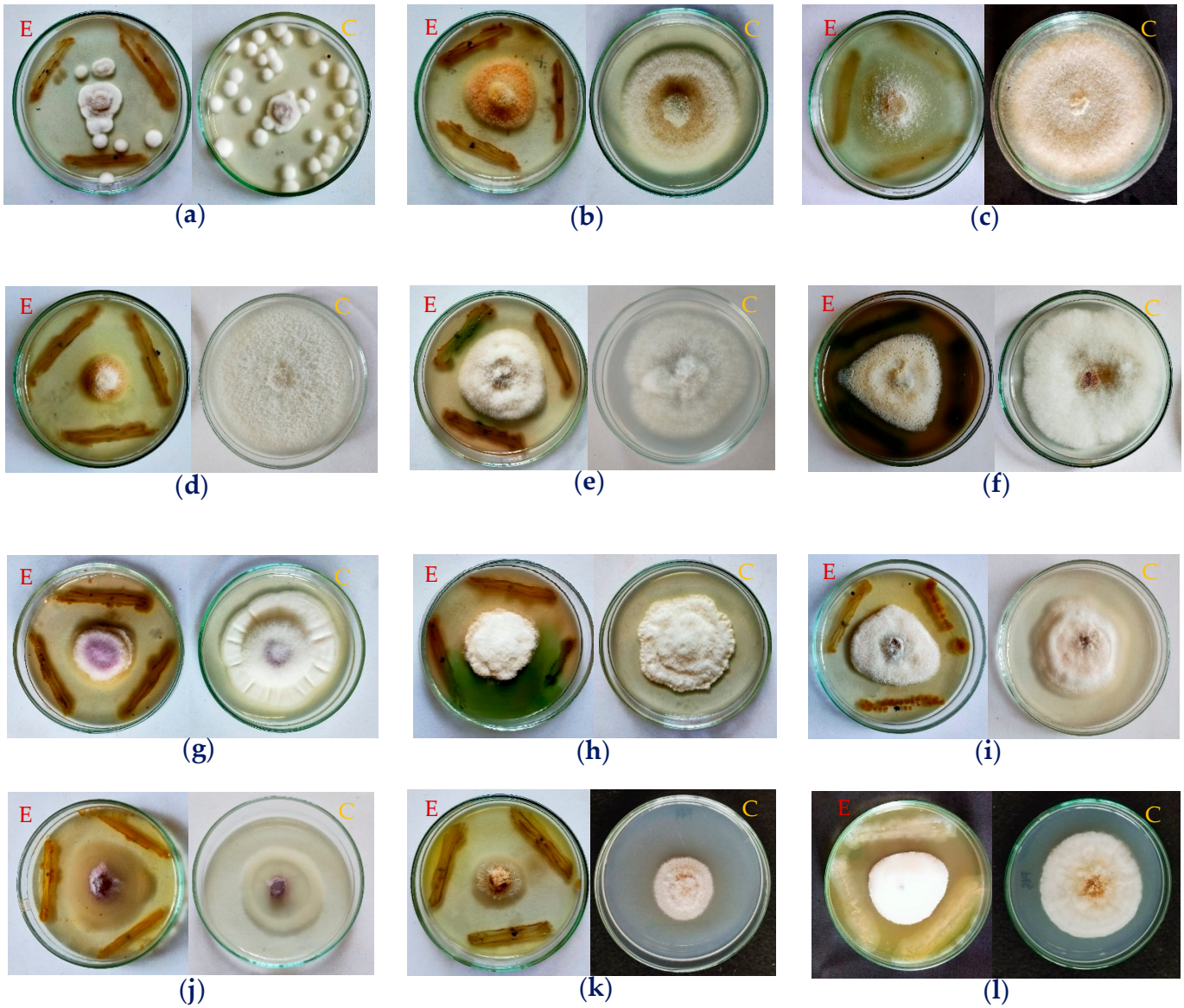

Figure 6. Evaluation of anti-fungal activity of *Pseudomonas aeruginosa* TUN03 against various plant plant-pathogen strains, including *Purpureocillium lilacinum* F01 (**a**), *Fusarium solani* F02 (**b**), *F. solani* F03 (**c**), *F. solani* F04 (**d**), *C. gloeosporioides* F05 (**e**), *F. incarnatum* F06 (**f**), *G. butleri* F07 (**g**), *Pestalotiopsis mangiferae* F08 (**h**), *F. incarnatum* F09 (**i**), *F. oxysporum* F10 (**j**), *Neonectria* sp. F11 (**k**), and *F. incarnatum* F15 (**l**). A mycelial plug of growing fungal strain was placed into the center of the Petri dish containing PDA (potato D-glucose agar) medium and three lines of *Pseudomonas aeruginosa* TUN03 was streaked at a distance of 2 cm from the center of the surface of PDA medium in Petri dishes. These plates were then maintained at 28 °C for 5 d. The experimental and control dish are labeled as **E** and **C**, respectively.

Among these tested fungal strains, *Pseudomonas aeruginosa* TUN03 demonstrated the most efficient inhibition against *F. solani* F04 isolated from coffee roots with a high inhibition value of 70.51%, and this value was ranked as excellent (level a) based on Duncan's multiple range test. The strain TUN03 also showed inhibition against *F. solani* F02, *F. incarnatum* F06, and *G. butleri* F07, which were isolated and identified from Durio roots, citrus roots, and pepper roots, respectively, with potent inhibition values in the range of 41.67–50%, and ranked at levels b–d. *Pseudomonas aeruginosa* TUN03 showed moderate inhibitory effect against several fungal strains, including *F. solani* F03, *C. gloeosporioides* F05, *Pestalotiopsis mangiferae* F08 (30–35%), and weak inhibition of *F. incarnatum* F09, and *Neonectria* sp. F11 at a rate of 15.09–20.5%. *Pseudomonas aeruginosa* TUN03 was not effective against *F. oxysporum* F10 isolated from pepper roots. As shown in Figure 2A, *Pseudomonas aeruginosa* TUN03

significantly reduced the appearance of fungal colonies of *Purpureocillium lilacinum* F01, indicating more than 70% inhibition of this pathogenic fungus (the calculation was based on counting the colonies in experiment and control dishes). The spread of spores of this fungal strain around the surface of the Petri dish and the quick appearance of the colonies (Figure 1A) made it difficult to calculate the standard inhibition values based on the protocol presented by Ngo et al. 2020 [32] in the methods section.

4. Conclusions

In this study, we first assessed the potential use of rhizobacteria for biocontrol of Robusta coffee nematodes in the Central Highland of Vietnam. *Pseudomonas aeruginosa* TUN03 was screened and identified as the most effective strain. This selected bacterium significantly reduced the number of nematodes in soils and roots of coffee seedlings and was significantly effective in plant growth promotion in greenhouse tests. *Pseudomonas aeruginosa* TUN03 was found to produce multiple plant-promoting compounds and showed anti-fungal effects against various plant pathogen fungi that seriously damage some crops. Notably, this is the first report of the potent nematicidal effect of *Pseudomonas aeruginosa* against coffee nematodes J2 and its egg hatching, and the production of multiple plant-promoting compounds with high yield. Among these, the yield of GA3 was the highest, at 2702 μg/mL. TUN03 also displayed potency in biocontrol of various tested pathogen fungal strains. This work suggests, for the first time, that *Pseudomonas aeruginosa* TUN03 could be a potential biocontrol agent and a biofertilizer.

Author Contributions: Conceptualization, V.B.N.; methodology, V.B.N., A.D.N. and S.-L.W.; software, V.B.N., D.M.T. and D.N.N.; validation, A.D.N. and S.-L.W.; formal analysis, V.B.N., D.M.T., C.T.D., T.N.T., A.D.N., T.H.N., M.D.D., V.A.N., V.C.D. and S.-L.W.; investigation, V.B.N. and D.N.N.; resources, V.B.N. data curation, V.B.N., A.D.N. and S.-L.W.; writing—original draft preparation, V.B.N.; writing—review and editing, V.B.N., A.D.N. and S.-L.W.; visualization, V.B.N., A.D.N. and S.-L.W.; supervision, V.B.N., A.D.N. and S.-L.W.; project administration, V.B.N. and S.-L.W.; funding acquisition, V.B.N. and S.-L.W. All authors have read and agreed to the published version of the manuscript.

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