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Orchid-associated endophytic *Bacillus* mediates *Fusarium* suppression and promotes *in vitro* regeneration of banana plantlets via culture supernatant

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ABSTRACT The application of *Fusarium*-antagonistic endophytic bacteria with plant growth-promoting traits offers an effective method to enhance the success of banana plantlet tissue culture while combating Fusarium wilt disease caused by *Fusarium oxysporum* f.sp. *cubense* Tropical Race 4 (FocTR4) (VCG 01213). This study evaluates the endophytic bacterium AP3311, isolated from healthy banana roots in direct association with orchid roots. AP3311 exhibited strong antagonism toward FocTR4, hyphal colonization ability, and multiple growth-promoting activities, including phosphate solubilization, nitrogen fixation and auxin production. 16S rRNA gene sequencing identified that AP3311 belongs to the genus *Bacillus*, while metabarcoding analysis revealed that *Bacillus* species dominate the root microbiomes of both bananas and orchids. The bacterial supernatants stimulated root development and leaf growth *in vitro*. Metabolomic profiling indicated that antimicrobial compounds, together with plant growth regulators, promoted both root and shoot growth. Overall, the research demonstrates that *Bacillus* sp. AP3311 and its supernatants are valuable components in banana tissue culture, providing the dual benefits of plant growth promotion and effective disease control.

KEYWORDS Endophytic *Bacillus*; *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4; *In vitro* regeneration; Bacterial culture supernatant; Metabolomics

1. Introduction

Banana (*Musa* spp.) is a globally important fruit crop that is critically threatened by Fusarium wilt, caused by the soil-borne pathogen *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (Foc TR4) (Maryani et al. 2019), posing a serious challenge to sustainable production and global food security (Dita et al. 2018). Conventional chemical controls have been proven largely ineffective for long-term management due to the pathogen's persistent survival strategies as well as environmental and health concerns associated with agrochemical use (Ploetz 2015).

In vitro propagation remains an essential technique for the rapid multiplication of banana plants. However, tissue culture-derived plantlets often exhibit underdeveloped physiological and anatomical root functions and lack association with beneficial microbiome traits that increase their vulnerability to pathogenic infections (Soumare et al. 2021).

Endophytic bacteria, particularly *Bacillus* spp., have demonstrated strong potential as plant growth promoters and biological control agents. This potential is attributed to their ability to produce phytohormones (e.g., indole-3-acetic acid), solubilize phosphate, fix atmospheric nitrogen, and secrete diverse antimicrobial secondary metabolites (Nakkeeran et al. 2021).

Applying bacterial culture supernatants allows for a microbe-free strategy for delivering bioactive compounds, thus supporting plant growth and defense mechanisms while decreasing the potential risks linked to the introduction of live microbes, which could lead to unknown interactions (Naamala et al. 2022). Moreover, the elimination of bacterial cells facilitates a more direct evaluation of plant responses to chemical signals, thereby allowing researchers to differentiate effects due to soluble metabolites. However, despite the increasing number of studies concerning the application of *Bacillus* supernatants in promoting plant growth (Msimbira et al. 2023), their impact on banana *in vitro* tissue culture regeneration, specifically root and shoot induction, remains largely unexplored, and their antagonistic activity against FocTR4 has yet to be clearly demonstrated. Furthermore, the bioactive metabolites responsible for these responses within a tissue culture environment are not yet fully characterized.

This study investigated the endophytic *Bacillus* isolate AP3311, isolated from banana roots growing in close

proximity to orchid root during the greenhouse acclimatization of tissue culture-derived plantlets under shade condition. This spatial proximity may facilitate the incorporation of beneficial bacteria into the rhizosphere, potentially augmenting root microbiome functionality. The effects of its culture supernatant on the regeneration of banana tissue culture plantlets and its biocontrol activity against FocTR4 were evaluated. An integrated approach combining molecular identification, microbiome composition analysis through metabarcoding, and comprehensive metabolomic profiling was used to elucidate the bioactive mechanisms and to explore the practical potential of AP3311 in improving banana micropropagation quality and contributing to sustainable Fusarium wilt management.

2. Materials and Methods

2.1. Isolation of endophytic bacteria

2.1.1 Maintenance and root sampling of banana plantlets

Tissue cultured-derived banana plantlets were maintained under shaded and humid conditions during greenhouse acclimatization. Shading was provided by *Vanda* sp. orchids, initially collected from a forest habitat and later cultivated in a greenhouse in Karanggayam, Sleman, Yogyakarta. The orchids were mounted on pieces of tree fern stem, positioned above the banana plantlets. This arrangement contributed to the establishment of banana root systems in close spatial proximity to the aerial roots of the *Vanda* orchid within a shared microenvironment (Supplementary Figure S1). Banana plantlets were maintained under these conditions for about one year before root sampling for the isolation of endophytic bacteria.

2.1.2 Endophytic bacterial consortium screening

Banana roots, including both primary and secondary roots from the rhizosphere in proximity to the *Vanda* orchid roots, were thoroughly rinsed under running tap water for 2–3 min, followed by gentle massaging for 1 min in the presence of 0.1% Tween 20. Surface sterilization was performed with a modified protocol by Slama et al. (2019). Briefly, the roots were sequentially immersed in 70% ethanol for 3 min and in 1% sodium hypochlorite for 1 min, and finally rinsed three times with sterile distilled water for 1–2 min per rinse. The sterility of the final rinse was confirmed by plating 100 µL of the rinse water onto Nutrient Agar (NA) and checking for microbial growth. Nutrient Agar served as a general-purpose medium to selectively isolate culturable, fast-growing endophytic bacteria for subsequent functional assays.

The sterilized roots were aseptically cut into 1 mm segments and placed on NA Petri dishes. Following incubation at 30 °C for two days, morphologically distinct colonies originating from the same root segment were co-subcultured to establish preliminary mixed cultures, designated as consortia AP31, AP32, AP33, and AP34. These consortia were subsequently used for screening assays, including antagonism against FocTR4 (Supplementary Figure S1).

2.1.3 Isolation and selection of endophytic bacterial isolates

According to the initial screening results, consortium AP33 exhibited the highest antagonistic activity against FocTR4, thus leading to its selection for further purification and strain-level characterization. The bacterial consortium AP33 was subcultured onto fresh NA plates to obtain pure cultures. From this process, 11 distinct bacterial isolates were purified and preserved for further evaluation (Tsalgatidou et al. 2023). These isolates were subjected to the same antagonistic assay previously applied to the original consortium to identify strains that retained or enhanced antifungal activity (Supplementary Figure S2), followed by plant growth promotion (PGP) assays. Based on superior performance, four candidate isolates, AP334, AP336, AP339, and AP3311, were selected, with AP3311 emerging as the most effective antagonist and plant growth-promoting strain, particularly in indole-3-acetic acid (IAA) production, and therefore chosen for downstream applications and detailed characterization.

2.2. Antagonism test against FocTR4: direct and indirect inhibition

2.2.1 Direct inhibition

To evaluate bacterial–fungal interactions under varying growth conditions, potato dextrose agar (PDA) and nutrient agar (NA) facilitated the vegetative growth of FocTR4, whereas mung beans agar (MBA) was employed to enhance sporulation-related conditions. The antagonistic activity of bacterial consortia (AP31, AP32, AP33, and AP34) and bacterial isolates (AP3301- AP3311) against FocTR4 (VCG 01213), obtained from the Research Center for Biotechnology, Universitas Gadjah Mada) was assessed *in vitro* on these media, namely PDA, NA, and MBA. A 5 mm fungal agar plug was placed at the center of each Petri dish, and 10 µL aliquots of bacterial suspension were streaked at four equidistant points around the fungal plug, each positioned 2.5 cm from its edge. Negative controls consisted of fungal plugs without bacterial inoculation. Each bacterial consortium was tested in triplicate on PDA, NA, and MBA, whereas individual isolates were tested in triplicate on PDA.

Plates were incubated at 30 °C for 8 days. Fungal growth inhibition was calculated using the equation: Inhibition percentage = $(1-a/b) \times 100\%$, where a is the shortest distance between the edge of fungal growth facing the bacterial colonies and the edge of bacterial growth facing the fungus, and b is the corresponding distance in the control plate (Slama et al. 2019).

2.2.2 Indirect\volatile inhibition

The inhibitory effects of volatile organic compounds (VOCs) produced by endophytic bacterial isolates (AP334, AP336, AP339, AP3311) were evaluated using a modified method described by Wu et al. (2019). For each treatment, 10 µL of bacterial suspension at a concentration of 1×10^7 CFU mL^{-1} was inoculated onto the surface of PDA medium on the lower plate; the upper plate containing PDA was inoculated with a 5 mm diameter plug of FocTR4 mycelium. The two plates were sealed together with Parafilm to form a double chamber system, maintaining approximately 15 mm between medium surfaces. Controls consisted of double chambers without bacterial inoculation. All plates were incubated at room temperature for 7–10 days, with three replicates per treatment. Mycelial growth inhibition was assessed by visual observation and/or measuring colony diameter of FocTR4 at the end of incubation, and data were statistically analyzed to determine significant differences among treatments.

2.3. DNA isolation, 16S rRNA amplification, and metabarcoding

2.3.1 DNA isolation

Microbiome profiling of banana and *Vanda* orchid roots was performed by extracting total genomic DNA from culturable bacteria isolated from root samples. The roots were surface-sterilized, homogenized, and inoculated into Nutrient Broth (Himedia, India), followed by incubation at 30 °C with shaking at 150 rpm for 12 h to enrich culturable bacterial populations. Bacterial cells were then harvested, and genomic DNA was extracted using the Presto™ Mini gDNA Bacteria Kit (Cat. No. GBB100, Geneaid, Taiwan) according to the manufacturer's instructions with minor modifications to optimize yield and purity. DNA concentration and purity were assessed using a Nanodrop spectrophotometer (Thermo Scientific). Genomic DNA from the selected isolate AP3311 was extracted separately using the same procedure for culture-dependent analyses.

2.3.2 PCR amplification of 16S rRNA gene

The nearly full-length 16S rRNA gene was amplified using universal bacterial primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). PCR reactions consisted of a total volume of 25 µL containing 12.5 µL of PowerPol 2X PCR Mix (Cat.No.:RK20718, AB Clonal, USA) 1× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each primer, and 20 ng of genomic DNA template. Thermocycling conditions included an initial denaturation at 95 °C for 45 s; 30 cycles of 95 °C for 30 s, 54 °C for 30 s, 72 °C for 20 s; and included final extension at 72 °C for 5 min. PCR products were verified on 1% agarose gels.

2.3.3 Amplicon sequencing of isolate AP3311

The purified PCR product from isolate AP3311 was sequenced by PT Widya Teknologi Hayati, Widya Life Sciences, Yogyakarta, Indonesia, using standard Sanger sequencing protocols. Resulted sequences were analyzed using NCBI BLAST (Basic Local Alignment Search Tool) to identify the closest matching bacterial taxa by aligning against the GenBank database. This analysis confirmed the taxonomic identity of isolate AP3311.

2.3.4 Nanopore 16S rRNA gene metabarcoding and sequencing

Approximately 20 ng of DNA per PCR reaction was amplified to generate 1500 bp amplicons spanning the full length of the 16S rRNA gene using primers 27F and 1492R. All sequencing was performed by the Integrated Genomic Factory, Faculty of Biology, Universitas Gadjah Mada using the Oxford Nanopore Technologies (ONT) 16S Barcoding Kit (SQK 16S024) protocol.

2.4. Plant growth promoting characterization

2.4.1 Phosphate solubilization

A 10 µL of bacterial cultures was spot inoculated onto Pikovskaya agar medium (Himedia, India), containing five grams per liter of calcium phosphate and incubated at 30 °C for 2–3 days. The clear zone around the spotted bacterial culture indicated positive phosphate solubilization activity (Tsalgatidou et al. 2023).

2.4.2 Nitrogen fixation

The endophytic bacteria capable of nitrogen fixation were able to grow on Jensen's medium (Reang et al. 2022). A 10 µL of bacterial isolate was streaked on a Jensen's medium (Himedia, India) plate. Next, the cultures were incubated at 30 °C for 24 h.

2.4.3 IAA production

One colony of bacterial isolate was inoculated in a five mL of Tryptic Soy Broth medium (Himedia, India) supplemented with L-tryptophan (1 mg/mL) at pH 7, and then incubated at 150 rpm and 30 °C for 6 and 12 h. Subsequently, the cultures were centrifuged at 13000 rpm for 10 min, 4 °C. One mL of supernatant was mixed with Salkowski reagent (50 mL $HClO_4$ 35% and 1 mL $FeCl_3.6H_2O$ 0.5M) in a ratio of 1:2. Positive reaction of IAA was detected by the pink color formation. Quantitative analysis of IAA was performed by spectrophotometry at λ 530 nm (De Fretes et al. 2021)

2.5. Motility and hyphal colonization

2.5.1 Bacterial motility

A total of 10 µL of bacterial suspension was streaked on NA medium. The bacteria were incubated at 37 °C for 1 h. Bacterial movement was observed using an inverted

Nikon Diaphot 300 microscope equipped with an Optilab Advance Plus (Miconos) at 200× magnification.

2.5.2 Hyphal colonization

The dual culture between endophytic bacteria and FocTR4 was about 30 days old when the edges of the bacterial colony and the mycelium came into contact. Hyphal colonization was assessed microscopically and characterized as the observable association of bacterial cells with fungal hyphae, encompassing attachment to or movement along the hyphal surface at the colony interaction zone. Observations were made using an inverted Nikon Diaphot 300 microscope equipped with an Optilab Advance Plus (Miconos) at 200× magnification.

2.6. Preparation and application of culture supernatant

2.6.1 Cell-free supernatant preparation

AP3311 was cultured in Tryptic Soy Broth medium (Himedia, India) for 6 and 12 h at 30 °C with 150 rpm shaking. After incubation, the cultures were centrifuged and filtered through 0.22 µm membranes to obtain sterile cell-free supernatants. The supernatant from 12 h of incubation was used in the next experiment. The 12 h incubation period was chosen due to bacterial densities attaining 10^5–10^7 CFU mL^{-1} and the observable production of IAA associated with favorable root induction responses.

2.6.2 Supernatant effect on plant *in vitro* regeneration

Roots and leaves of *in vitro* banana plantlets were excised using a sterile blade, leaving the basal pseudostem region as the explant. The explants were then cultured on Murashige and Skoog (MS) medium supplemented with cell-free supernatant obtained from a 12 h culture of AP3311 at concentrations of 0.1%; 1%; and 10% (v/v). For each treatment, 10 explants were used per replicate. Root induction frequency, numbers of root and leaf per explant, were recorded at 5 and 10 days after induction. Cultures were maintained at 25 ± 2 °C under a 12 h light/12 h dark photoperiod. Previously, three banana cultivars, namely 'Raja Bagus', 'Cavendish Giant', and 'Cavendish Siger', were cultured on MS medium supplemented with low-dose IAA, 0.001 ppm, for 10 and 15 days under the same culture conditions. Data were analyzed using one-way analysis of variance (ANOVA), followed by Duncan's Multiple Range Test (DMRT) at $P < 0.05$

2.6.3 Supernatant effect on mycelial growth

A five mm agar plug containing FocTR4 was taken from a five day old culture on PDA. The plug was placed centrally on a fresh PDA plate (90 mm diameter). Sterile agar wells (5 mm diameter) were bored into the agar at the four cardinal points, 3 cm from the plug. Into each hole, 20 µL of the following treatments were added: (i) supernatant of the *Bacillus* isolate at 1%, 10% and 100% (v/v); (ii) Carbendazim fungicide solution at 10 ppm, 100 ppm and 1000 ppm. Plates were incubated at 28 °C in the dark. Mycelial growth was measured daily for 7 days as colony diameter, determined by measuring the distance across the colony through the center of the fungal plug along two perpendicular axes and calculating the mean value. All assays were performed in triplicate.

2.6.4 Supernatant effect on hyphae morphology

A 10 mL of Mung Bean Broth (4 gr of Mung Bean in 1 L of distilled water) was inoculated with a five mm mycelial plug of FocTR4 at 30 °C, 150 rpm for 24 h. A 300 µL of spore suspension contains 10^5 CFU spore mL^{-1} in dH_2O, and the addition of supernatant (0, 4.69, 9.38, 18.75, 37.5, 75, 150, and 300 µL) in a sterilized 96-well plate was incubated at 30 °C in dark conditions for 12 h. The hyphae morphology of the germinated spore of FocTR4 was observed using inverted microscopy Nikon Diaphot 300 and Optilab Advance Plus (Miconos). Microscopic observation focused on spore germination, early hyphal development, and visible morphological alteration compared with control.

2.7. Metabolomic profiling of culture supernatant

Untargeted metabolomic analysis was performed using a Thermo Scientific™ Vanquish™ Horizon UHPLC system coupled to an Orbitrap™ Exploris 240 high-resolution mass spectrometer (Thermo Fisher Scientific, Germany). Each biological condition represented AP3311 culture supernatant collected at 6 h and 12 h of incubation, with four biological replicates pooled for metabolomic profiling. Chromatographic separation was conducted on a Thermo™ Accucore™ C18 column (100 × 2.1 mm, 2.6 µm) at 40 °C, with mobile phases consisting of (A) water and (B) acetonitrile, both containing 0.1% formic acid. A gradient from 5% to 90% B over 16 min, held for 4 min, and returned to 5% B (total run time 25 min) was applied at a flow rate of 0.3 mL/min, and 5 µL of sample was injected.

The HRMS was operated in Full MS/dd-MS^2 mode with polarity switching, a scan range of 70–800 m/z, mass resolutions of 60,000 (Full MS) and 22,500 (dd-MS^2), mass accuracy of ≤ 5 ppm, and collision energies of 30, 50, and 70. H-ESI ion source was used with spray voltages of +3.5 kV and −2.5 kV. Other parameters were as follows: sheath gas at 35 AU, auxiliary gas at 7 AU, sweep gas at 1 AU, an ion transfer tube temperature at 300 °C, and a vaporizer temperature at 320 °C.

Samples (500 µL) were diluted 1:1 with HPLC-grade methanol, filtered (0.2 µm nylon filter), and transferred to HPLC vials. Data processing and compound identification were performed using Compound Discoverer 3.3, with spectral matching against mzCloud and masslist databases (e.g., Arita Flavonoids, LIPID MAPS, Natural Products Atlas), complemented by ChemSpider cross-searching (KEGG, BioCyc, FDA, etc.).

3. Results and Discussion

3.1. AP33 as an antagonistic consortium

This study began with the isolation of endophytic bacteria through three sequential screening stages. The first stage focused on selecting an antagonistic endophytic bacterial consortium, consisting of AP31, AP32, AP33, and AP34 (Table 1), based on their ability to inhibit the *Fusarium oxysporum* f.sp. *cubense* Tropical Race 4 (FocTR4). Antagonistic activity was evaluated using dual-culture assays (Supplementary Figure S2) and volatile compound inhibition tests (Supplementary Figure S3). Consortia AP33 and AP34 inhibited FocTR4 mycelial growth by 51.10% and 45.03%, respectively, on PDA medium (Table 1). Volatile inhibition was only observed for AP33, making it the primary candidate for further isolation. The support of active vegetative growth of FocTR4 by PDA indicates that the observed inhibition on this medium is due to antagonistic activity targeting mycelial expansion, while no inhibition was noted under sporulation-promoting conditions (MBA).

3.2. AP3311 as an antagonistic isolate against FocTR4 and Plant Growth-Promoting Bacterium

Purification of AP33 yielded 11 distinct isolates (AP331-AP3311). In the second screening stage, four isolates (AP334, AP336, AP339, and AP3311) exhibited > 50% inhibition against FocTR4, with AP3311 showing the highest inhibition (55.51%) (Figure 1, Supplementary Figure S5, and Figure S6). AP3311 also suppressed fungal growth via volatile compounds (Supplementary Figure S4), confirming its potential as a superior antagonist.

3.3. Four endophyte isolates demonstrate plant growth-promoting abilities

The four most antagonistic isolates were further evaluated for plant growth- promoting bacteria (PGPB) traits, including phosphate solubilization, nitrogen fixation, and IAA production. All isolates demonstrated these activities to varying degrees (Table 2).

AP334 and AP336 had the highest baseline level of IAA production without the addition of L-Tryptophan precursor, ranging from 1.795 to 1.863 ppm, which significantly increased to 6.162–7.071 ppm upon addition of

TABLE 1 Inhibitory effect of endophytic consortium bacteria against *F. oxysporum* f.sp. *cubense* TR4 mycelial growth across three growth media potato dextrose agar (PDA), nutrient agar (NA), and mung beans agar (MBA). Different letters within the same column indicate significant differences at $P < 0.05$ according to Duncan's Multiple Range Test (DMRT).

Consortium	Inhibition Zone (%)			Volatile Inhibition Diameter (mm)
	PDA	NA	MBA	
Control	-	-	-	33.41 ± 0.09^{b}
AP31	0	0	0	29.90 ± 0.82^{c}
AP32	0	0	0	45.77 ± 0.32^{a}
AP33	51.10 ± 3.71^{a}	17.68 ± 2.19^{b}	0	25.53 ± 1.50^{d}
AP34	45.03 ± 1.95^{b}	34.28 ± 1.67^{a}	0	47.20 ± 0.70^{a}

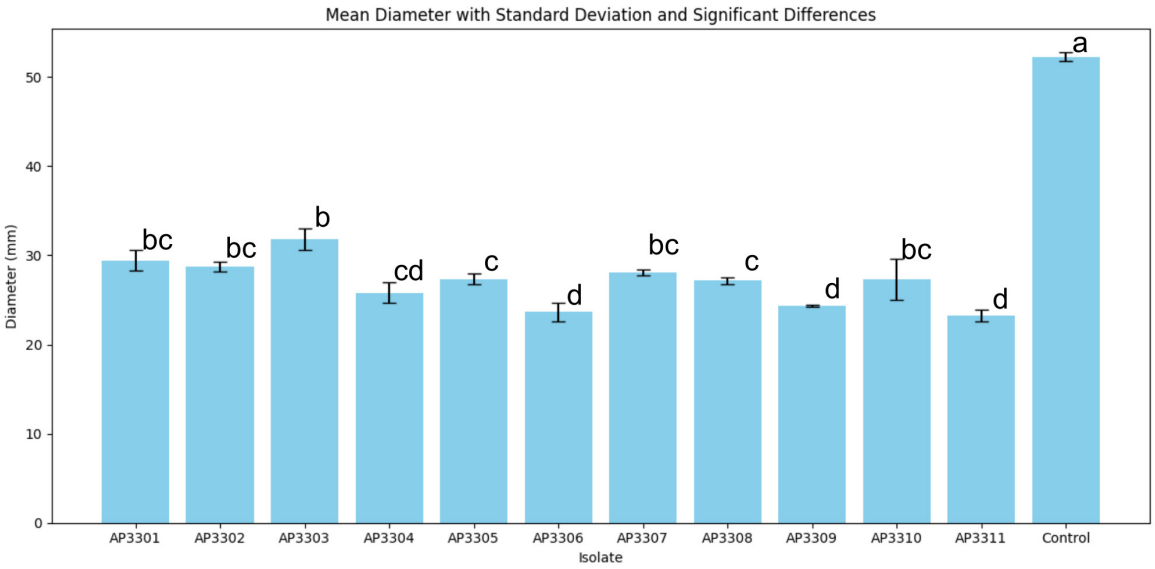

FIGURE 1 Direct inhibition of *Fusarium* mycelial growth by 11 endophytic bacterial isolates from banana roots, observed at eight days post-inoculation. The assay was conducted on 90 mm diameter plates using the dual culture method. Different letters above the bars indicate significant differences at $P < 0.05$ according to Duncan's Multiple Range Test (DMRT).

TABLE 2 Plant growth-promoting activities and antagonistic mechanisms of four selected endophytic bacterial isolates. Antagonistic mechanism assessed included hyphal colonization, direct inhibition, and volatile inhibition against *F. oxysporum* f.sp. *cubense* Tropical Race 4 (FocTR4). The symbol (+) indicates the presence of activity.

Isolate	Phosphate Solubilization	N Fixation	IAA	Hyphal Colonization	Motility	Direct Inhibition (%)	Volatile Inhibition (%)
AP3304	+	+	+	+	+	50.61	6.84
AP3306	+	+	+	+	+	54.73	4.12
AP3309	+	+	+	+	+	53.4	2.24
AP3311	+	+	+	+	+	55.51	26.02

100 ppm L-Tryptophan (Table 3). All isolates were motile and capable of hyphal colonization, essential traits for effective root association. Based on combined antagonistic and growth-promoting properties, AP3311 was selected for further study.

3.4. Taxonomic Identification of AP3311

16S rRNA gene sequencing revealed ≥ 99% similarity of AP3311 to *Bacillus velezensis* / *B. amyloliquefaciens* (Supplementary Figure S7b), consistent with previous reports of *Bacillus* dominance in healthy banana roots and *Vanda* orchid roots (Supplementary Figure S7a).

TABLE 3 Comparison of IAA production by endophytic bacterial isolates with and without Tryptophan supplementation. Data represent the mean ± standard deviation. Different letters within the same column indicate significant differences at $P < 0.05$ according to Duncan's Multiple Range Test (DMRT).

Treatments	IAA Concentration (ppm) Mean ± SD
AP3304	1.86 ± 0.01^{c}
AP3304 + Tryptophan	6.16 ± 0.01^{b}
AP3306	1.79 ± 0.01^{c}
AP3306 + Tryptophan	7.07 ± 0.01^{a}
AP3309	0.47 ± 0.01^{d}
AP3309 + Tryptophan	6.48 ± 0.01^{b}
AP3311	1.22 ± 0.01^{cd}
AP3311 + Tryptophan	7.00 ± 0.01^{a}

TABLE 4 The influence of bacterial supernatant on *in vitro* root regeneration of 'Raja Bagus' banana plant. The assay was conducted for 10 days after inoculation (dai). Data presented are mean ± standard deviation. Different letters within the same column indicate significance differences at $P < 0.05$ according to Duncan's Multiple Range Test (DMRT). T0: MS + 20 gr/L sucrose, at 10 dai. T1: S 0.1%; T2: S 1%; T3: S 10%; T4: Agar + 20 gr/L sucrose + S 0.1%; T5: MS + 20 gr/L sucrose + 0.01 ppm IAA.

Treatments	Regeneration (%)	Number of Roots
T0	100	1.88 ± 0.61^{c}
T1	100	3.00 ± 0.82^{ab}
T2	100	3.50 ± 0.58^{a}
T3	100	1.25 ± 0.5^{c}
T4	100	2.50 ± 0.71^{bc}
T5	100	3.50 ± 0.58^{a}

3.5. Effect of AP3311 supernatant on in vitro Banana plantlets regeneration

Although AP3311's supernatant contained relatively low levels of IAA, its potensials were evaluated to stimulate root regeneration in three banana cultivars 'Raja Bagus', 'Cavendish Giant', and 'Cavendish Siger'. Supplementation with 0.001 ppm synthetic IAA induced root formation by day 5 in 60% of 'Cavendish Giant' and 'Cavendish Siger' plantlets, compared with 10% in 'Raja Bagus'. By day 10, the low-dose IAA treatment achieved 100% root induction in both 'Cavendish' cultivars, compared with 40% in controls (Supplementary Table S1).

The effect of AP3311 bacterial supernatant on root induction in the 'Raja Bagus' variety was further tested. Supplementation of supernatant at 0.1, 1, and 10 percent per 100 mL of Murashige and Skoog media, as well as 0.1 percent in agar + sucrose (20 g/L) media, enhanced root induction, observed from day 5 (Table 4 and Supplementary Figure S9). In addition to root induction, shoot growth was observed as an additional indicator of the positive effects of bacterial metabolites, indicating the presence of other compounds that contribute to stimulating organ development.

3.6. Antifungal Activity of AP3311 Supernatant

Undiluted AP3311 supernatant inhibited FocTR4 mycelial growth by 18.9% after 5 days, exceeding the inhibition achieved by carbendazim (13.6% at 1000 ppm), but actively declined by day 10 (Supplementary Table S2).

Incubation of 100 spores with the supernatant for 12 h caused abnormal germinating hyphae, characterized by a rounded and bulging morphology at intercalary zones and hyphal tips, in contrast to the straight, filamentous hyphae in the control, indicating morphological disruption (Supplementary Figure S8).

3.7. Bioactive metabolites in AP3311 supernatant

After processing the LC-HRMS data, a refined subset of metabolites was chosen for subsequent visualization and interpretation. Metabolites presented in Figure 2 were prioritized according to reliable annotation via database matching (mzCloud and masslist databases), established biological significance in relation to plant growth promotion or antifungal activity, and consistent detection across various incubation conditions. Metabolite features characterized by low annotation confidence, redundant signals,

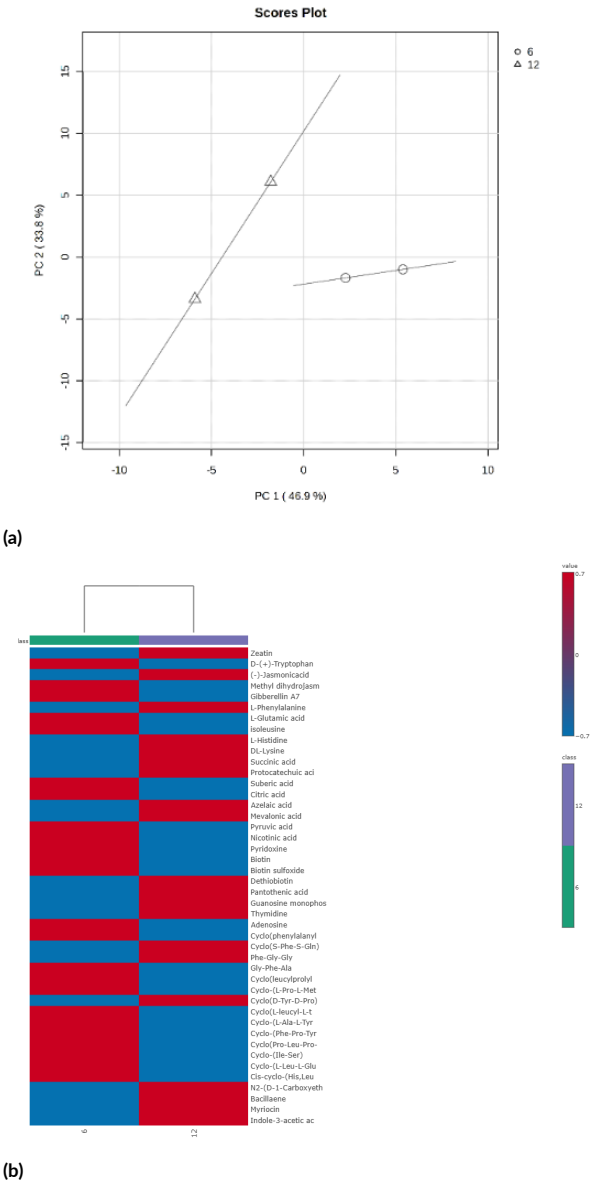

FIGURE 2 Clustering and profile analysis of endophytic bacterial supernatant metabolites based on incubation time. Principal component analysis (PCA) showing the grouping of metabolite profiles from 6 to 12 h of incubated culture (a). Heatmap visualizing the relative expression profiles of the identified metabolites (b).

or uncertain biological relevance were omitted from the Figure 2 but preserved in the comprehensive LC-HRMS dataset for reference purposes.

LC-HRMS analysis identified phytohormones (IAA, zeatin, gibberellin, and jasmonic acid) and vitamins (pyridoxine, pantothenic acid, nicotinic acid, and biotin) essential for plant growth and development (Figure 2). Antimicrobial compounds, including bacillaene, were also detected, along with unidentified metabolites.

3.8. Discussions

The use of antibiotics in tissue culture to prevent contamination can inadvertently eliminate beneficial endophytic bacteria in bananas, particularly those that promote plant growth and enhance resistance to biotic and abiotic stresses (Quambusch et al. 2014). Consequently, restoring endophytic bacteria in tissue culture-derived plantlets has become an important strategy for sustainable Fusarium wilt control.

Selecting appropriate endophytic bacteria for bioprimingbanana plantlets is a crucial step. The chosen strains must combine antagonistic activity against FocTR4 with the ability to promote plant growth (Shen et al. 2022). In this study, endophytic bacteria were isolated from tissue culture-derived banana plantlets that had been planted for one year. While the plantlets were initially free of endophytes, exposure to the environment enabled natural microbial colonization.

Endophytic bacteria are known to enter root tissues, proliferate, and cycle back into the rhizosphere, a process described as the rhizophagy cycle (White et al. 2018). Here, banana roots growing adjacent to *Vanda* orchid roots were used as the inoculum source, as orchids are thought to enrich the rhizosphere with beneficial endophytes. The direct contact observed between banana and orchid roots (Supplementary Figure S1) suggests that these orchid-associated endophytes may transfer into banana rhizosphere, strengthening its microbial community and biocontrol capacity.

Endophytic isolation began with consortium screening for antagonism against FocTR4, followed by purification of the most inhibitory consortium members (Table 1, Supplementary Figure S2 and Figure S3). Unlike conventional approaches that typically yield hundreds of isolates (Posada et al. 2024), this targeted method reduced isolate numbers and accelerated the identification of promising candidates.

Among four consortia tested, AP33 and AP34 displayed antagonistic activity. However, volatile inhibition assays revealed that AP34 not only failed to suppress FocTR4 mycelium growth but also stimulated it, whereas AP33 consistently inhibited pathogen development. From AP33, eleven morphologically distinct isolates were obtained. Four isolates—AP334, AP336, AP339, and AP3311—demonstrated > 50% colony inhibition, a level considered effective. However, inhibition levels were slightly lower than those reported for *Bacillus siamensis* sp. QN2MO-1 ± 60% (Zhang et al. 2024). The four isolates exhibited strong suppression of *Fusarium* growth on PDA, accompanied by aerial hyphae formation, a feature associated with conidiospore dispersal (Choi et al. 2024). Notably, AP3311 induced fewer aerial hyphae than the other isolates (Supplementary Figure S6), highlighting its potential as a biocontrol candidate.

These four isolates were further characterized for plant growth promotion traits, including indole-3-acetic acid (IAA) production, phosphate solubilization, nitrogen fixation, motility, and hyphal colonization. AP3311 emerged as the most promising strain, combining FocTR4 antagonism with plant growth promotion. Additional studies are needed to unravel its molecular mechanisms and broader PGP traits.

The phylogenetic tree derived from 16S rRNA sequences indicated minimal genetic divergence among *B. velezensis*, *B. amyloliquefaciens*, and related taxa, leading to unresolved clustering within the *B. subtilis* species complex. This emphasizes the restricted discriminatory capacity of the 16S rRNA gene for species-level resolution within this group and highlights the necessity for genome-based or multilocus strategies for establishing taxonomic classification.

16S rRNA analysis revealed that AP3311 shares ≥ 99% similarity with *B. velezensis* and *B. amyloliquefaciens*. This finding is consistent with metabarcoding profiles showing *Bacillus* dominance in healthy banana roots and *Vanda* orchids (Idiyatov et al. 2022). This observation indicates a possible ecological correlation and justifies the need for additional functional characterisation of AP3311, rather than suggesting causality. *Bacillus* endophytes are well known for producing antimicrobial compounds and phytohormones, supporting the dual role of AP3311. To confirm strain-level identity and explore biosynthetic gene clusters, whole-genome sequencing (WGS) will be conducted.

The supernatant of AP3311 contained IAA, sufficient to trigger root and leaf regeneration in excised banana plantlets. In *Arabidopsis*, wounding induces local auxin accumulation, initiating root development within 1–2 days through priming, initiation, patterning, and emergence. After priming, auxin interacts with cytokinin to promote this process, whereas excess gibberellin can impair auxin transport and delay root formation (Xu 2018). By day 5, banana explants treated with AP3311 supernatant showed enhanced root and leaf growth compared to controls, underscoring the importance of phytohormone synergy in tissue regeneration.

IAA production can be further enhanced through precursor supplementation or extended incubation. LC-HRMS analysis indicated increasing IAA concentrations at 6 and 12 h (Figure 2). By 12 h, cultures reached ~10^7–10^8 CFU mL^{-1}, the standard density for biopriming (Roslan et al. 2020). In addition, AP3311 effectively solubilized phosphate by secreting organic acids and phosphatases, a mechanism common among *Bacillus* and *Pseudomonas* species (Sun et al. 2024). Its nitrogen-fixing ability further enhances its value as a plant growth-promoting bacterium (PGPB), reducing dependence on synthetic fertilizers and supporting plant productivity (Bidabadi et al. 2021). With its three main functions—IAA production, phosphate solubilization, and nitrogen fixation—AP3311 represents a strong candidate for use as an eco-friendly biostimulant.

Root and leaf growth also facilitate bacterial colonization. Photosyntates from leaves are transported to roots and released as exudates, attracting PGPB that metabolize these compounds and secrete growth-promoting metabolites (Sun et al. 2017). AP3311 demonstrated both motility and hyphal colonization (Table 2), suggesting a strong ability to interact with plant tissues and participate in the rhizophagy cycle, ensuring the persistence of beneficial bacterial populations.

Antagonism against FocTR4 by AP3311 involves two main mechanisms: direct inhibition of mycelial growth and the production of volatile antagonistic compounds. Although short-term (12 h) supernatant assays showed limited inhibition (Supplementary Table S2), LC-HRMS metabolomics analysis successfully identified bacillaene, a potent antimicrobial metabolite (Figure 2b). *Bacillus* species are also known to produce macrolactin, bacillysin, and bacillobactin (Zhang et al. 2024). The low inhibition observed here is likely due to insufficient metabolite accumulation during short incubation. Previous studies showed stronger inhibition with extended culture times (72 h to 6 days), allowing metabolite buildup (Zhang et al. 2024; Vibha et al. 2025). Thus, optimizing culture period and conditions may enhance AP3311's antagonistic potential.

Morphological changes were also observed in *Fusar-*

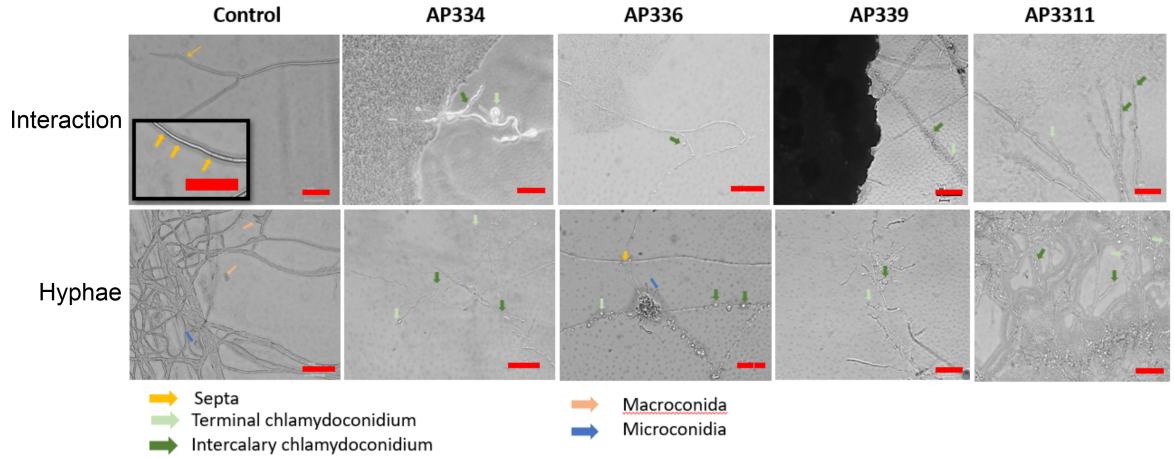

FIGURE 3 Antagonistic interaction between endophytic bacteria and *F. oxysporum* f. sp. *cubense* Tropical Race 4 (FocTR4). The figure shows direct colonization and induction of chlamydospore-like structures observed at the interface between the fungal mycelial edge and the bacterial colony. (Scale bar = 50 µm).

ium hyphae exposed to AP3311 supernatant, including swollen, thick-walled structures resembling chlamydospores (Supplementary Figure S8). Such stress-induced modifications, absent in controls, are commonly triggered by antagonistic microbes and their metabolites (Djemouai et al. 2023). These responses became more apparent at higher supernatant concentrations, indicating a dosage-related effect on hyphal morphology, although under the short incubation period used, the effect was primarily associated with growth stress rather than complete inhibition of spore germination. Furthermore, in direct contact assays, the bacterial isolate AP3311 was observed to colonize the FocTR4 hyphae, further inducing profound morphological alterations, including the formation of chlamydospore-like structures (Figure 3). Similar alterations have been linked to bacterial volatiles and cell wall damage (Liu et al. 2017). Supernatants of other *Bacillus* strains, such as *B. velezensis* CBMB205, also induce chlamydospore-like structures (Vibha et al. 2025), supporting the hypothesis that AP3311 disrupts fungal growth both structurally and metabolically.

The comparison of the three antagonistic modes reveals that direct bacterial–fungal contact is the most effective in suppressing FocTR4, leading to significant inhibition of mycelial growth, decreased aerial hyphae formation, and notable morphological disruption. Volatile-mediated inhibition contributed to the suppression of fungal growth without direct contact, although the effect was less pronounced. In contrast, treatment with cell-free supernatant resulted in stress-related morphological alterations accompanied by minimal growth inhibition during brief incubation periods. These findings indicate that AP3311 suppresses FocTR4 via multiple, complementary mechanisms rather than through a singular bioactive compound.

Taken together, these results highlighted the dual role of AP3311 as a biocontrol agent and a plant growth promoter. Its activities are mediated by phytohormone production, nutrient mobilization, antimicrobial metabolites, and rhizosphere colonization. Biopriming with AP3311 represents a promising and sustainable strategy for Fusarium wilt management in banana. Future research should focus on elucidating molecular interaction mechanisms, conducting experiments on the pathogen-inoculated banana plantlets, leading to field trials, especially to observe the capability of the bacterial endophytes to survive and manage the functions within the interaction among other microbes.

4. Conclusions

Endophytic isolate AP3311 exhibited >99% 16S rRNA sequence similarity to *B. velezensis* and *B. amyloliquefaciens*. This strain demonstrated a dual function (i) effective biocontrol agent against FocTR4, achieving ≈55% mycelial growth inhibition, and (ii) bio-enhancement of lateral root formation and leaf growth when applied at 0.1–10% culture supernatant. Metabolomic profiling identified bacillaene, a group of lipopeptides, and phytohormones (IAA, zeatin, gibberellin), elucidating a potential mechanism underlying its antagonistic and plant growth-promoting activities. These findings position AP3311 as a promising, sustainable biocontrol and biofertilizer candidate for supporting early banana plantlet establishment prior to acclimatization. Nevertheless, validation through multi-location field trials, whole-genome sequencing, and regulatory safety assessments are essential to confirm its efficacy and commercial feasibility under practical agricultural conditions.

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Authors' contributions

ABS, SS, YAP, and BSD designed the study. ABS carried out the laboratory work, analyzed data, and wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests related to this study.

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