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Exploration and evaluation of native bacterial antagonist against *Fusarium* wilt disease (*Fusarium oxysporum* f.sp. *cubense* Tropical Race 4) in banana (*Musa* spp.)

AMARESH HADIMANI[1], R THANGAVELU[2]*, KANTHARAJU V[1], AMRUTA S BHAT[1], DADAPEER A PEERJADE[3], SUHASINI JALAWADI[3] and MAHANTESHA NAIKA B N[1]

ICAR-National Research Centre for Banana, Tiruchirappalli, Tamil Nadu 620 102, India

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ABSTRACT

An experiment was conducted during 2022 and 2023 at ICAR-National Research Centre for Banana, Tiruchirappalli, Tamil Nadu to isolate and assess native rhizospheric and endophytic bacterial strains for their potential biocontrol activities against *Fusarium oxysporum* f.sp. *cubense* Tropical Race 4 (*Foc* TR4) causing wilt in banana (*Musa* spp.). A total of 75 bacterial strains, comprising 50 endophytic and 25 rhizospheric isolates, were obtained from various parts of banana plants and their rhizospheric soils. The experiment was laid out in a completely randomized block design (CRBD) with six treatments and five replications. *In-vitro* screening revealed that *Bacillus subtilis* (Ple-3 and Kr-2), *Bacillus subtilis* (Ykmr-6), *Pseudomonas fluorescens* (Por-4), and *Bacillus velezensis* (Toe2) significantly inhibited spore germination (93.43–100%) and mycelial growth (67–78%) of *Foc* TR4. These isolates demonstrated biocontrol activities, including HCN production, chitinase and protease activity, as well as phosphate solubilization, thereby enhancing their biocontrol potential. Molecular analysis confirmed their identity as *Bacillus* spp. and *Klebsiella* spp. and sequences were deposited in GenBank (OR472994 to OR472998). Combined soil application of *Bacillus subtilis* (Ykmr-6) + *Pseudomonas fluorescens* (Por-4) reduced internal wilt score in tissue-cultured banana plants. A lowest internal wilt score 0.8 (average of two seasons) was observed to be most effective in reducing internal wilt scores in tissue-cultured banana plants cv. Grand Nain. The study highlights the potential of bacterial strains, *Bacillus subtilis* (Ple-3 and Kr-2), *Bacillus subtilis* (Ykmr-6), *Pseudomonas fluorescens* (Por-4), and *Bacillus velezensis* (Toe2) as biocontrol agents, providing an alternative to chemical methods in managing *Fusarium* wilt in bananas and paving the way for integrated disease management strategies in banana cultivation.

Keywords: Banana, Bacterial biocontrol agents, Characterization, Disease management, *Fusarium oxysporum* f.sp. *cubense* TR4, Wilt

Bananas (*Musa* spp.) originated in Indo-china and South-east Asia (Simmonds 1962) and have spread globally to tropical and subtropical regions. As the food crop ranked fourth in economic significance, after rice, wheat, and maize. Asia remains the predominant banana-producing region, with a volume share of 51.80%. *Fusarium* wilt, a major disease affecting banana crops, likely invented in South-east Asia and was first stated in Australia in 1876 (Ploetz and Pegg 2000). Cavendish bananas are generally unaffected to *Foc* race 1, which devastated the cv. Gros Michel (AAA) variety, but they are susceptible to tropical race 4 (TR4) which is causing severe epidemic in various banana-growing countries including India (Thangavelu *et al.* 2019). The worldwide banana industry remained stable up to the emergence of *Fusarium* wilt Race 4 (R4) in the 1990s, which severely affected Cavendish bananas. The onset of *Fusarium* wilt TR4 was initially identified in Cavendish plantations in Taiwan in 1970, later spreading to Indonesia and Malaysia in the early 1990s. In 2015, TR4 was indetified in Cavendish banana cultivars Robusta and Grand Nain in Bihar, India (Thangavelu *et al.* 2019). Current methods for managing fungal diseases include cultural, chemical and biological approaches, each with its drawbacks, such as fungicide residues, phytotoxicity and resistance development. Biocontrol agents (BCAs), including endophytes, *Trichoderma* spp., *Pseudomonas* spp., and *Bacillus* spp., have undergone thorough investigation for their potential in disease management. These bioagents operate through diverse modes of action, such as stimulating phytohormone production, competing with pathogens for essential nutrients and activating plant immunity (Singh

[1]Kittur Rani Channamma College of Horticulture, University of Horticultural Sciences, Bagalkot, Karnataka; [2]ICAR-National Research Centre for Banana, Tiruchirappalli, Tamil Nadu; [3]University of Horticultural Sciences, Bagalkot Karnataka. *Corresponding author email: rtbanana@gmail.com

2014). Integrating biological control agents (BCAs) that operate through various mechanisms can enhance biocontrol effectiveness through additive or synergistic effects (Parnell *et al.* 2016, De Vrieze *et al.* 2018). Here, we hypothesize that combined application of biological control agents through the utilization of antagonistic bacteria such as *Bacillus* and *Pseudomonas* spp. could offer a safe, effective, and eco-friendly alternative for the management of disease. This study aimed to isolate and evaluate native bacterial biocontrol agents with several traits against *Foc* TR4.

MATERIALS AND METHODS

Isolation and maintenance of the fungal pathogen: *Fusarium oxysporum* f.sp. *cubense* (VCG 01213/16, Tropical Race 4) was isolated from wilt-affected Cavendish banana samples (25.63243, 87.27612, Falka village, Katihar, Bihar, India) and purified via single conidium isolation (Riker and Riker 1936). The cut pieces of infected corm samples were surface sterilized with 0.1% $HgCl_2$ for 30 sec rinsed five times with sterile distilled water, dried for 20 min, and incubated on quarter-strength PDA media at 28°C for 5–7 days. The cultures were maintained on PDA slants at 25ºC and stored at 4ºC.

Isolation of rhizospheric and endophytic bacteria: 1 g of rhizospheric soil from various banana cultivars was mixed with 100 ml of sterile distilled water in a 250 ml flask and shaken at 120 rpm for 10 min. Then, 1 ml of the supernatant was serially diluted up to 10^{-5}. 1 ml from each, 10^{-4}, and 10^{-5} dilution was placed into sterilized petri plates with 15 ml of King's B (KB) agar medium at 45°C, mixed, and incubated at 28°C for 2 days. Endophytic bacteria were isolated from banana roots, corms, stems, and petioles collected from the experimental farm of ICAR-National Research Centre for Banana, Tiruchirappalli, Tamil Nadu (Supplementary Table 1). The samples were washed, surface-sterilized with ethanol and sodium hypochlorite, rinsed with sterile distilled water, and the rinse water was plated on TSA to confirm sterilization. Tissues were macerated, serially diluted, and 10^{-3}, 10^{-4}, and 10^{-5} dilutions were plated on KB agar, incubated at 28°C for 3 days. Bacterial colonies from rhizospheric and endophytic origin were purified and stored at -80°C as glycerol stock (Singh *et al.* 2022).

Evaluation of endophytic and rhizospheric bacterial isolates for multiple biocontrol activity under in vitro *condition*: The bacterial isolates were evaluated for various biocontrol actions against *Foc* (VCG01213/16). These activities included conidium germination (CSFT 1943), inhibition of mycelial growth in dual culture plates, and volatile production (Dennis and Webster 1971). Antifungal properties were tested using the agar well diffusion method (Mehmood *et al.* 1999). Additionally, the production of fungal cell wall-degrading enzymes, such as proteases (Simbert and Krieg 1994) and chitinase (Renwick *et al.* 1991), siderophore (Schwyn and Neilands 1987) and HCN production, were assessed. Furthermore, the plant growth-promoting capabilities of the bacterial isolates were evaluated, including the production of phosphate solubilisation indole acetic acid (IAA) (Gaur 1990) and (Brick *et al.* 1991)

Evaluations of bacterial antagonists against Foc TR4 under glass house conditions: The methodology of Vidhyasekaran and Muthamilan (1995) was followed for multiplication and formulation of bacterial antagonists. Briefly, potent endophytic and rhizospheric bacterial isolates were cultured in nutrient broth at 25ºC for 48 h. 1 kg of talcum powder was mixed with 10 g of Carboxy Methyl Cellulose (CMC), adjusted to *p*H 7 using calcium carbonate, and autoclaved. After cooling, 400 ml of bacterial suspension (9×10^9 cfu/ml) was mixed with the talc, air-dried to 35% moisture content, and stored in polypropylene bags at 28±2ºC. A completely randomized block design (CRBD) with six treatments and five replications each was used. Inoculated and uninoculated tissue culture plants (cv. Grand Nain) served as controls. Rhizospheric and endophytic bacterial antagonists were applied at 50 g/plant, followed by 30 g of *Foc* TR4 sand/maize culture after 10 days. 3–4 months after planting, plant growth parameters, including plant height, pseudostem girth, and leaf area and internal wilt score (vascular discoloration) were recorded using INIBAP's Technical Guidelines Number 6 (Carlier *et al.* 2002).

Identification of bacterial antagonists by 16S rRNA sequencing: DNA was isolated from pure bacterial cultures using (Moore *et al.* 1987) method. Briefly, 1 ml of overnight culture was taken and centrifuged at 15,000 rpm for 20 min. The supernatant was discarded and the pellet was re-suspended in 567 µL Tris-EDTA extraction buffer [(10 mM

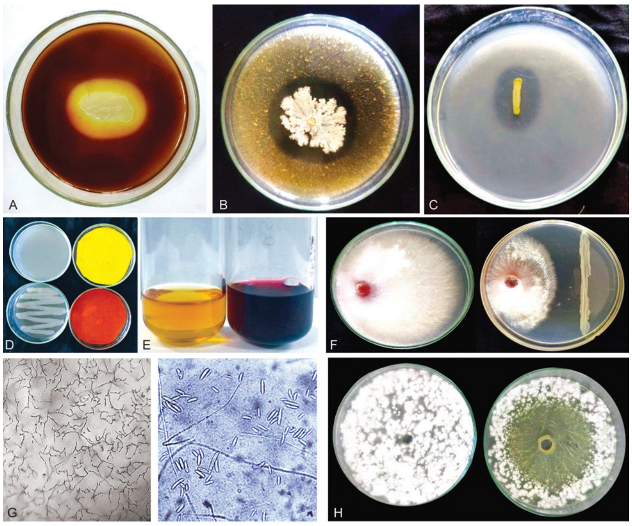


Fig. 1 *S*creening of endophytic and rhizospheric bacterial isolates against *Foc* TR4 for multiple traits under *in vitro* condition. A–B, Pectinase and Protease production by the isolate *Bacillus subtilis* (Ykmr-6); C–D, Phosphate solubilisation and HCN production by the isolate *Pseudomonas fluorescens* (Por-4); E, IAA production by the isolate *Bacillus subtilis* (Kr-2); Mycelia growth inhibition of *Foc*; F–G, Dual culture and spore germination inhibition assay by the rhizospheric *Pseudomonas fluorescens* (Por-4); H, Agar well diffusion by the rhizospheric isolate *Bacillus subtilis* (Kr-2).

Tris-HCl, 1 mM sodium ethylenediamine acetic acid (*p*H 8.0) and 30 µL, 10% sodium dodecyl sulphate (SDS)]. Subsequently, 3 µL of proteinase K 20 mg/ml was added and incubated for 1 h at 37ºC. A 100 µL volume of sodium chloride (NaCl) was added and mixed thoroughly and 80 µL of cetyltrimethyl ammonium bromide/sodium chloride mix (1% CTAB/0.7 M NaCl) was added and incubated for 10 min at 65°C. After incubation, an equal volume of phenol/chloroform/isoamyl alcohol (23:24:1) was added and the mixture was centrifuged for 5 min at 15,000 rpm. The aqueous phase was transferred to a sterile 1.5 ml micro centrifuge tube and an equal volume of chloroform/isoamyl alcohol (24:1) was added from which the aqueous phase was once again obtained. The DNA was then precipitated with 0.6 volume of isopropanol. Pellets were then washed with ice cold 70% ethanol and air dried at room temperature (28 ± 2ºC). The DNA pellet was resuspended in 100 µL TE buffer and the samples were stored at –20ºC. The mixture was processed with phenol/chloroform/isoamyl alcohol, and DNA was precipitated using isopropanol. It was then washed with ethanol, rehydrated in TE buffer, and stored at -20ºC.

PCR amplifications: The 16S rRNA gene from effective bacterial isolates was amplified using primers 27F and 1492R in a 25 µL reaction mixture. Thermal cycling included initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 52°C for 45 sec and 72°C for 1 min, with a final extension step at 72°C for 8 min. PCR products were separated on 1.5% agarose gel, visualized, excised, purified, and sequenced. Sequences were assembled, compared to GenBank using BLAST, aligned with Clustal W, and a Neighbor-Joining phylogenetic tree was constructed using MEGA7 with 1000 bootstrap replicates.

Statistical analysis: Data were statistically analyzed using software WASP-Web Agri Stat Package developed by ICAR Research Complex for Goa, Ela, and Old Goa. All data were first subjected to analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Screening of endophytic and rhizospheric bacterial isolates against Foc TR4: Among 50 endophytic and 25 rhizospheric bacterial isolates from various banana germplasms, two endophytic (*Bacillus subtilis* Ple-3, *Bacillus subtilis* Kr-2) and three rhizospheric (*Bacillus subtilis* Ykmr-6, *Pseudomonas fluorescens* Por-4, *Bacillus velezensis* Toe2) were effective against *Foc* TR4 *in vitro*. These isolates demonstrated 93.43–100% inhibition of spore germination and 67–78% inhibition of mycelial growth in dual plate assays, while agar well diffusion assays showed inhibition (Fig. 1) ranging from 30–100%. They also produced HCN, chitinase, and protease, known for controlling *Fusarium* wilt in bananas (Yadav *et al.* 2021).

Bacillus subtilis Ple-3, *Bacillus subtilis* Ykmr-6, and *Pseudomonas fluorescens* Por-4 effectively solubilized phosphate (1.26-5 cm lytic zone) (Table 1). These findings align with Saravanan *et al.* (2003), Thangavelu and Gopi (2015) who reported similar biocontrol activity against *Foc* by inhibiting the mycelial growth, spore germination

Table 1 *In vitro* screening of rhizospheric bacterial isolates for their multiple traits against *Fusarium oxysporum* f.sp. *cubense* Tropical Race 4 (VCG 01213/16)

Treatment	NCBI genBank accession number	Spore germination Inhibition (%)	Mycelia growth inhibition (%) Dual culture plate assay	Antifungal assay	Volatile production	HCN production	Siderophore production	IAA production (µg/ml)	Chitinase production (lytic zone in mm)	Protease production (lytic zone in mm)	Phosphate solubilization (lytic zone in cm)
Bacillus subtilis (Ple-3)	OR472994	94.66^{c} (76.66)	67.00^{c} (58.06)	30.00^{d} (33.16)	31.33^{b} (34.03)	-ve	-ve	1.80^{d}	0.00^{d}	0.00^{c}	1.26^{c}
Bacillus subtilis (Ykmr-6)	OR472995	100.00^{a} (88.83)	67.33^{bc} (55.15)	40.00^{c} (39.21)	49.33^{a} (44.61)	+ve	+ve	1.00^{e}	4.93^{a}	15.00^{a}	2.83^{b}
Pseudomonas fluorescens (Por-4)	OR472996	100.00^{a} (88.83)	78.00^{a} (54.95)	70.00^{b} (56.83)	46.0^{a} (42.70)	+ve	+ve	3.71^{c}	3.90^{b}	5.33^{b}	5.00^{a}
Bacillus subtilis (Kr-2)	OR472997	93.43^{c} (75.28)	72.00^{b} (62.04)	100.00^{a} (88.83)	21.00^{c} (27.22)	+ve	-ve	15.73^{a}	0.00^{d}	5.00^{b}	0.00^{d}
Bacillus velezensis (Toe2)	OR472998	96.66^{b} (79.59)	68.00^{bc} (55.57)	32.00^{d} (35.04)	27.67^{b} (31.72)	-ve	-ve	5.15^{b}	2.16^{c}	0.00^{c}	0.00^{d}
Control (*Foc* TR4 alone)	OR468256	0.00^{d} (1.17)	0.00^{d} (1.17)	0.00^{e} (1.17)	0.00^{d} (1.17)	-ve	-ve	0.00^{f}	0.00^{d}	0.00^{c}	0.00^{d}
S.Em±	-	0.60	1.52	2.20	1.22	-	-	0.21	0.18	0.63	0.33
CD (P=0.01)	-	2.60	6.62	9.55	5.29	-	-	0.92	0.80	2.75	1.43

Values are mean of three replications. Figures within parentheses are arc sine transformed values. Mean ratings a column that are followed by the same letter are not significantly different according to LSD at $P \leq 0.01$. +, Positive result; -, Negative result.

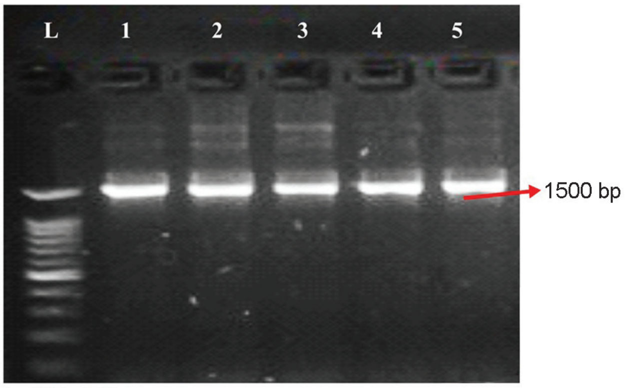
besides solubilisation of phosphorus, IAA and HCN production.

Evaluation of bacterial antagonists under pot culture conditions: In pot culture evaluations against *Fusarium* wilt TR4 in tissue-cultured banana plants (cv. Grand Nain), the rhizospheric *Pseudomonas fluorescens* (Por-4) was found to be most effective by recording an internal wilt score of 2. Endophytic *Bacillus subtilis* (Ple-3), *Bacillus velezensis* (Toe2), rhizospheric *Bacillus subtilis* (Ykmr-6) and *Bacillus subtilis* (Kr-2), each scored 3.0. *Foc*-inoculated plants scored 5.0, while uninoculated controls scored 0 (Supplementary Table 2). Among the combinations tested, the results showed that combined soil application of rhizospheric bacteria, *Bacillus subtilis* (Ykmr-6) + *Pseudomonas fluorescens* (Por-4) recorded the lowest internal wilt score 0.6 and 1 in season one and two respectively (Table 2). This is in accordance with the findings of Thangavelu and Gopi (2015) who reported the combined application of endophytic bacteria *P. putida* (C4r4) + rhizospheric *B. cereus* (Jrb1); recorded total suppression (100%) of *Fusarium* wilt under glass house conditions. Similarly, Wang *et al.* (2023) concluded that *Streptomyces* strain XY006 successfully colonized banana plantlets and suppressed the incidence of Fusarium wilt of banana (FWB), with a biocontrol efficacy of up to 87.7% under pot culture conditions. These findings also align with those of Saravanan *et al.* (2003), who identified *Pseudomonas fluorescence* strain Pfm as the most effective biocontrol agent against *Fusarium* wilt disease under pot culture condition. The success of *Pseudomonas fluorescens* (Por-4) aligns with recent findings that *Pseudomonas* spp. are potent biocontrol agents due to their production of antibiotics, siderophores, and hydrogen cyanide (HCN), which inhibit pathogen growth and enhance plant defense responses (De Vrieze *et al.* 2022). Compared to *Foc*-inoculated control plants, the bacterial treatments increased plant height up to 41.50%, girth up to 84.74% and leaf area up to 110.55% (Table 2). The increase in leaf area suggests enhanced photosynthetic capacity, contributing to plant vigour and productivity. Beneficial bacteria like *Bacillus* spp. and *Pseudomonas* spp. produce phytohormones such

Table 2 Evaluation of combined application of endophytic and rhizospheric antagonistic bacterial isolates against *Foc* TR4 under pot culture condition

Treatment	Height (cm)		Girth (cm)		Leaf area (cm^2)		Internal score (0–5 scale)	
	S1	S2	S1	S2	S1	S2	S1	S2
Bacillus subtilis (Ple-3) + *Bacillus subtilis* (Ykmr-6)	21.20^{f} (12.77)	33.60^{cd} (27.95)	7.64^{bc} (36.43)	11.08^{ab} (79.87)	381.76^{bc} (46.20)	769.60^{abc} (108.04)	2.00^{b}	1.00^{d}
Bacillus subtilis (Ple-3) + *Pseudomonasfluorescens* (Por-4)	21.40^{ef} (13.83)	36.80^{a} (40.14)	6.14^{ef} (9.64)	11.38^{a} (84.74)	313.44^{ef} (20.04)	778.88^{ab} (110.55)	3.00^{b}	3.40^{bc}
Bacillus subtilis (Ple-3) + *Bacillus subtilis* (Kr-2)	20.80^{f} (10.64)	37.16^{a} (41.51)	6.40^{e} (14.29)	10.64^{bc} (72.73)	318.88^{e} (22.12)	844.80^{a} (128.37)	3.00^{b}	3.00^{c}
Bacillus subtilis (Ple-3) + *Bacillus velezensis* (Toe2)	22.20^{def} (18.09)	36.20^{ab} (37.85)	6.70^{de} (19.64)	9.64^{g} (56.49)	338.56^{cde} (29.66)	709.76^{bc} (91.87)	3.00^{b}	3.40^{bc}
Bacillus subtilis (Ykmr-6) + *Pseudomonas fluorescens* (Por-4)	26.60^{a} (41.49)	36.00^{ab} (37.09)	8.60^{a} (53.57)	10.34^{cde} (67.86)	493.12^{a} (88.85)	750.72^{abc} (102.94)	0.60^{c}	1.00^{d}
Bacillus subtilis (Ykmr-6) + *Bacillus subtilis* (Kr-2)	22.90^{bcde} (21.81)	29.80^{e} (13.48)	7.50^{bc} (33.93)	11.08^{ab} (79.87)	364.16^{bcde} (39.46)	553.60^{d} (49.65)	3.00^{b}	3.00^{c}
Bacillus subtilis (Ykmr-6) + *Bacillus velezensis* (Toe2)	23.00^{bcd} (22.34)	31.88^{de} (21.40)	6.30^{e} (12.50)	9.72^{efg} (57.79)	375.68^{bcd} (43.87)	655.04^{cd} (77.08)	3.00^{b}	3.60^{bc}
Pseudomonas spp. (Por-4) + *Bacillus subtilis* (Kr-2)	24.40^{b} (29.79)	35.88^{ab} (36.63)	7.90^{b} (41.07)	10.32^{cde} (67.53)	412.96^{b} (58.15)	731.84^{abc} (97.84)	3.00^{b}	4.00^{ab}
Pseudomonas spp. (Por-4) + *Bacillus velezensis* (Toe2)	23.80^{bc} (26.60)	32.80^{cd} (24.90)	7.20^{cd} (28.57)	9.80^{def} (59.09)	411.52^{b} (57.60)	671.36^{bcd} (81.49)	3.00^{b}	4.00^{ab}
Bacillus subtilis (Kr-2) + *Bacillus velezensis* (Toe2)	22.80^{cde} (21.28)	34.48^{bc} (31.30)	6.20^{ef} (10.71)	10.52^{bcd} (70.78)	323.52^{de} (23.90)	746.88^{abc} (101.90)	3.00^{b}	3.40^{bc}
Un-inoculated control	21.00^{f} (11.70)	34.60^{bc} (31.76)	6.60^{de} (17.86)	9.20^{fg} (49.35)	336.64^{cde} (28.92)	659.52^{cd} (78.29)	0.00^{c}	0.00^{e}
Foc TR4 alone	18.80^{g} (0.0)	26.26^{f} (0.00)	5.60^{f} (0.0)	6.16^{h} (0.00)	261.12^{f} (0.00)	369.92^{e} (0.00)	5.00^{a}	4.60^{a}
SEm±	0.55	0.74	0.22	0.25	18.84	41.83	0.24	0.26
CD (*P*=0.01)	2.11	2.85	0.87	0.96	71.49	158.68	0.92	1.00

Values are mean of five replications. Figures in parentheses are per cent increase over *Foc* TR4 alone inoculated control plants. Mean ratings within a column that are followed by the same letter are not significantly different according to LSD at $P \leq 0.05$. S1, Season 1; S2, Season 2.



L : 100 bp ladder
1 *Bacillus subtilis* (Ple-3) 3 *Pseudomonas* spp. (Por-4) 5 *Bacillus velezensis* (Toe2)
2 *Bacillus subtilis* (Ykmr-6) 4 *Bacillus subtilis* (Kr-2)

Fig 2. PCR amplification of effective bacterial antagonists using 16s rRNA primers.

as indole-3-acetic acid (IAA), promoting root elongation and plant growth (Glick 2012). These growth enhancements were attributed to high biocontrol actions of the bacterial isolates, which included inhibition of spore germination and mycelial growth inhibition, volatile metabolite and hydrogen cyanide (HCN) production, siderophore secretion, lytic enzyme activity including protease production, and phosphate solubilization. Further supporting these findings, Yadav *et al.* (2021) reported that the individual application of *Bacillus licheniformis* CSR-D4 significantly lowered the percentage of disease incidence to 10% when plants faced challenges with *Foc* TR4 under pot culture conditions. Thangavelu and Gopi (2015) showed that applying four rhizospheric bacterial isolates individually reduced mean internal wilt severity scores to 2–3, compared to a score of 5.0 in *Foc*-inoculated control plants in pot culture conditions.

Identification of bacterial antagonists by 16S rRNA sequencing: PCR amplification using universal primers (23F and 1492R) generated 1500 base pair amplicons (Fig. 2) for the 16S rRNA region of two endophytic (Ple-3 and Toe2) and three rhizospheric (Ykmr-6, Por-4, and Kr-2) bacterial isolates. Sequence analysis revealed significant homology with *Bacillus subtilis*, *Bacillus velezensis*, *Pseudomonas fluorescens* and *Bacillus subtilis*, respectively.

The bacterial isolate sequences were submitted to GenBank with accession numbers OR472994 to OR472998. The study's findings align with Sun *et al.* (2011), Li *et al.* (2012) and who identified *Bacillus* strains using 16S rDNA sequences. Similarly, Guinazu *et al.* (2013) characterized *Pseudomonas fluorescens* using PCR. Phylogenetic analysis grouped related strains on shorter branches, indicating recent common ancestors, with bootstrap values showing confidence levels. For instance, *Bacillus subtilis* strains formed clusters with high bootstrap values, indicating close genetic relationships, while *Escherichia coli* was more distantly related (Supplementary Fig. 1). The study identified five bacterial isolates (two endophytic and three rhizospheric) with notable biocontrol activity against *Foc* TR4 in bananas. Future directions include exploring synergistic effects by combining these isolates with other fungal biocontrol agents and or agronomic practices and investigating their mechanisms of action to optimize their use and effectiveness.

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