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Original Research Article

In vitro Antagonistic Effects of Plant Growth-Promoting *Bacillus* spp. Isolated from *Talinum fruticosum* on *Fusarium oxysporum*

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ABSTRACT

This study investigated the in vitro antagonistic effect of plant growth-promoting rhizobacteria, Bacillus spp., isolated from Talinum fruticosum, a plant known for its therapeutic and dietary value against the phytopathogenic fungus Fusarium oxysporum. The antagonistic potential of these Bacillus spp. was assessed through in vitro assays, including dual-culture techniques and antimicrobial tests. Results revealed that of all the bacterial isolates identified via cultural and biochemical methods which were then confirmed through molecular analysis with over 99% sequence similarity, Bacillus subtilis exhibited the most significant plant growth-promoting (PGP) traits, testing positive for phosphate solubilization, indole acetic acid production, ammonia production, and nitrogen fixation. This highlights its potential as an effective agent for enhancing plant growth in rhizosphere applications. The in vitro antagonistic potential of Bacillus subtilis against the pathogenic fungus Fusarium oxysporum, with Bacillus subtilis showing a peak inhibition rate of 46.43% at 48 hours. These findings underscore the promising role of Bacillus subtilis as a biological regulator agent that can militate against Fusarium oxysporum, offering a sustainable alternative to chemical control methods for managing plant diseases. Further field trials are recommended to validate these in vitro results and to explore the practical applications of these isolates in sustainable agriculture.

1. INTRODUCTION

Plant health is essential for food security, and constitutes a major pointer to safe and sustainable food systems as well as boost farmers' incomes (Dlamini and Akanmu et al., 2022). Farmers lose around 10-23 percent of

their produces to fungal infection yearly, and extra 10 percent is lost after-harvest regardless of extensive use of antifungals (Nuwamanya and Runo et al., 2023). This has led to rapid decrease in crop yield and poses a serious threat to food security, given that a substantial portion of the world's food production is derived from crops susceptible to these fungal infections likely caused by *Fusarium oxysporum* (Ekwomadu and Mwanza, 2023).

Fusarium oxysporum is a pervasive soil-borne pathogen responsible for significant losses of crops worldwide, comprising of vegetables, cereals, and ornamental plants (Enagbonma and Imade et al., 2023). The pathogen invades the plant's vascular system, leading to wilting, yellowing, and often death of the plant, thereby affecting crop harvest and value (Ismaila and Ahmad et al., 2023). The management of Fusarium wilt has traditionally relied on chemical fungicides, which, while effective, pose environmental hazards and contribute to the improvement of resistant pathogen strains (Okorska and Dąbrowska et al., 2023). Consequently, there is an emergent interest in developing sustainable and ecological friendly alternatives to chemical control (Amoo and Enagbonma et al., 2021). Among these alternatives, plant growth-promoting bacteria (PGPB) have gained attention due to their ability to suppress soil-borne pathogens via numerous underlying principles like induction of systemic resistance, antimicrobial compound productions, and competition in herbaceous perennial plant like *Talinum fruticosum* (Enagbonma and Fadiji et al., 2023, Santos and do Nascimento et al., 2024).

Talinum fruticosum, commonly known as waterleaf, is a leafy vegetable widely grown in tropical and subtropical regions (Enagbonma and Solomon, 2024). Despite its nutritional and medicinal importance, the rhizosphere of *Talinum fruticosum* remains relatively unexplored for beneficial microorganisms that could contribute to sustainable agriculture (Enagbonma and Momoh, 2024). The rhizosphere is a dynamic environment where plant roots interact with a diverse microbial community, including PGPB that can enhance plant growth and offer protection against pathogens (Babalola and Enagbonma, 2024). This study focuses on isolating and characterizing PGPB from the rhizosphere of *Talinum fruticosum* and evaluating *Bacillus* sp. antagonistic activity against *Fusarium oxysporum.*

Bacillus sp., recognized for their ability to produce antibiotics and other antimicrobial compounds (Ajuna and Lim et al., 2024), may offer a promising biological control method against *Fusarium oxysporum* (Ajilogba and Babalola et al., 2013). By investigating the interactions between *Bacillus* isolate and the pathogen, this research aims to identify effective strains that can deter the growth and action of *Fusarium oxysporum*. Understanding these antagonistic relationships could pave the way for developing eco-friendly biocontrol agents, reducing reliance on chemical fungicides, and contributing to sustainable agricultural practices (Elanchezhiyan and Keerthana et al., 2018). Thus, the aim of this study is to investigate the in vitro antagonistic effect of plant growth-promoting *Bacillus spp.,* isolated from *Talinum fruticosum* against *Fusarium oxysporum.*

2. MATERIALS AND METHODS

2.1. Study Area

Samples of rhizosphere soil from *Talinum fruticosum* were randomly collected from six different sites (SL1, SL2, SL3, SL4, SL5, and SL 6) at the Ekosodin, Benin City (Figure 1). The soils were mined from 0 - 15 cm depth within the *Talinum fruticosum* rhizosphere (Enagbonma and Fadiji et al., 2024). These samples were carefully placed in ice-cooled boxes, labeled appropriately, and conveyed to the research center and kept at 4°C for subsequent isolation, identification, and enumeration of the bacteria present in the *Talinum fruticosum* rhizosphere.

2.2. Bacterial Isolation

Soil samples from the rhizosphere were weighed and serially diluted in 10-fold steps (The formula for the dilution factor is given below in Equation (1) (Okoduwa and Enagbonma et al., 2022)). 0.01kg of soil were mixed with 90 ml of sterile saline, and 1 ml of this solution was further diluted by transferring to test tubes containing 9 ml of sterile water. From the fourth dilution tube, 0.1 ml was inoculated into sterilized Petri dishes, and nutrient agar with 1% fluconazole (to suppress fungal growth) was added. Bacterial cultures were prepared using the pour plate method, with replicates created for accuracy. Bacterial isolates were

characterized using morphological, biochemical, and molecular methods previously reported by Osayomwanbo and Imade et al. (2019). For molecular analysis, DNA was mined from *Talinum fruticosum* rhizosphere soils by employing the Nucleospin Soil kit, and bacterial strains were identified by 16S rDNA sequencing (Chen and Cai et al., 2014).

$$Dilution\ factor\ =\ \frac{final\ volume}{aliquot\ volume} \tag{1}$$

$$where\ final\ volume = aliquot\ volume\ (sample\ volume)\ + diluent\ volume$$

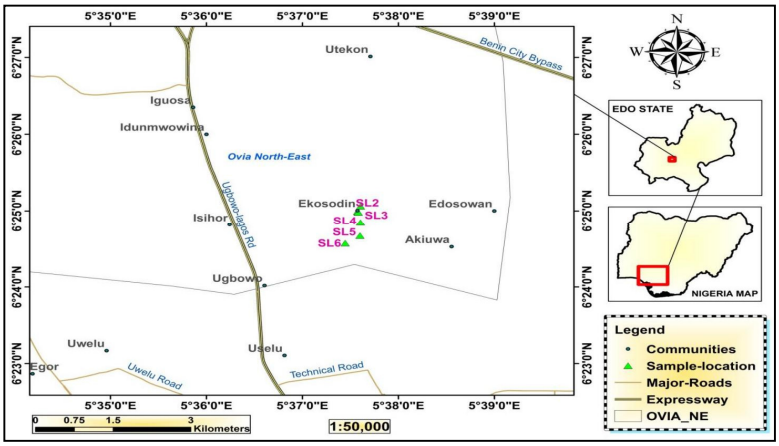

Figure 1: Geographical representation of sample location

2.3. Plant growth Promoting Characteristics of Bacterial Isolates

2.3.1. IAA production test

Indole-3-acetic acid (IAA) production screening involved culturing bacterial isolates in a liquid medium containing 500 mg/L L-tryptophan. These cultures were then reacted with Salkowski's reagent, which consists of 1 ml of 0.5 M FeCl3 solution and 50 ml of 35% perchloric acid, in tryptic soy broth (supplemented with 1 g/L MES hydrate, adjusted to pH 6). The cultures were incubated at 30°C for about 3 days on a rotary shaker. Post-incubation, the broth was centrifuged at 1200 x g for 15 minutes. For IAA detection, 1.0 ml of the supernatant was combined with 2.0 ml of Salkowski's reagent and left to incubate at room temperature for 25 minutes. The appearance of a pink color indicated the presence of IAA (Randive and Agnihotri et al., 2024).

2.3.2. Ammonia production test

Fresh bacterial cultures were introduced into 10 ml of nutrient broth and then incubated at 30°C for 2 days on a rotary shaker. Following the incubation period, 0.5 ml of Nessler's reagent was added to each sample. The formation of a yellow to brown color signified a positive result for ammonia production (Richard and Adekanmbi et al., 2018).

2.3.3. Phosphate solubilization test

The bacterial isolate was placed in the center of a sterile Pikovskaya agar plate and incubated for 3 days at 30°C. After the incubation period, the areas of phosphate solubilization surrounding the bacterial colonies were examined. The solubilization index was determined by calculating the ratio of the total halo diameter to the colony diameter (Wasoontharawat, 2017).

2.3.4. Ammonia production test

Freshly cultured bacteria were introduced into 10 ml of nutrient broth and incubated at 30°C for 2 days using a rotary shaker. After the incubation period, 0.5 ml of Nessler's reagent was added to each sample. A brown to yellow color change indicated the production of ammonia (Adebajo and Akintokun et al., 2021).

2.4. Antagonistic Activities of Plant Growth-Promoting Bacteria Against *Fusarium oxysporum*

The inhibitory effects of diffusible compounds on *Fusarium oxysporum* were assessed in vitro through dual culture assays. *Fusarium oxysporum*, isolated from the tomato rhizosphere, was cultured on Sabouraud dextrose agar (SDA) plates by placing a 7 mm disc from an actively growing fungal culture at the center of each plate. A 24-hour-old culture of bacterial strains was then streaked 2.5 cm away from the fungal disc. The plates were incubated at 28°C for 5 days. Additionally, *Trichoderma* was tested for its antagonistic effect against *Fusarium oxysporum*, serving as a comparison to evaluate the effectiveness of *Bacillus* in inhibiting *Fusarium* growth. Antifungal activity was assessed by measuring the inhibition zones around the bacterial colonies. Fungal growth inhibition was quantified using Equation 2 adapted from Adebajo and Akintokun et al. (2021), where R1 indicates the maximum radial fungal growth towards the bacterial antagonist, and R2 represents the distance between the fungal and bacterial inoculation points.,

$$\text{Inhibition (\%)} = \text{R1-R2/R1} \times 100 \quad (2)$$

2.5. Statistical Analysis

The whole research investigations were conducted in triplicate. The average for all measured parameters were calculated, along with the standard error and standard deviation.

3. RESULTS AND DISCUSSION

3.1. Cultural, Morphological and Biochemical Identifications of Bacteria Isolated from Soil Samples Collected from *Talinum fruticosum*

The cultural, morphological, and biochemical characterization confirmed the presence of *Bacillus* sp.*, E. coli, Alcaligenes faecalis* sp.*, Serratia* sp.*, Klebsiella* sp., *Pseudomonas* sp., and *Enterobacter* sp. (Table 1).

Table 1: Identification of bacteria isolated from *Talinum fruticosum* rhizosphere

Elevation	Flat	Raised	Flat	Flat	Flat	Raised	Raised
Margin	Entire	Entire	Undulate	Undulate	Undulate	Entire	Entire
Color	Cream	Lemon	Cream	Cream	Cream	Cream	cream
Shape	Circular	Circular	Irregular	Irregular	Irregular	Circular	Circular
Size	Small	Medium	Large	Large	Large	Medium	Medium
Gr. diff. agar	EMB	PCA	EMB	BCA	EMB	EMB	PCA
Colour	Pink	Green	Green	Straw	Pink	opaque	cream
Staining							
Gram stain	-	-	-	+	-	-	-
Cell type	Rod	Rod	Rod	Rod	Rod	rod	rod
Arrangement	disperse	Disperse	disperse	disperse	disperse	disperse	disperse
Color	Pink	Pink	Pink	purple	Pink	pink	pink
Spore staining	-	-	-	+	-	-	-
Biochemical							
KOH String Test	+	+	+	-	+	+	+
Catalase	+	+	+	+	+	+	+
Indole	-	-	+	-	-	-	-
Citrate	+	+	-	+	+	+	+
Oxidase	-	+	-	-	-	-	+
Motility	-	+	+	+	+	+	+
Urease	+	+	-	-	-	-	-
Identity	*Klebsiella* sp.	*Pseudomonas* sp.	*E. coli*	*Bacillus* sp.	*Enterobacter* sp.	*Serratia* sp.	*Alcaligenes* sp.

Plant diseases are among the primary constraints to agricultural productivity, with the *Fusarium* genus being one of the most destructive and economically significant groups of fungi (Ekwomadu and Mwanza, 2023). These pathogens severely impact both the quality and yield of crops worldwide (El Chami and El Chami et al., 2023). Employing beneficial microorganisms like the ones identified in Table 1 to suppress plant pathogens offers a sustainable and environmentally friendly alternative to chemical control methods (Imade and Babalola, 2021).

3.2. Molecular Identification of the Bacterial Isolates

Molecular analysis of soil samples from the rhizosphere of *Talinum fruticosum* corroborated the presence of these bacterial isolates. The bacterial isolates identified included *Bacillus subtilis* (99.91%), *Pseudomonas aeruginosa* (99.91%), *Serratia marcescens* (99.91%), *Escherichia coli* (100.00%) and *Enterobacter aerogenes* (100.00%) (Table 2).

Table 2: Molecular identification of the bacterial isolates

Code	Scientific Name	Max Score	Total score	Query Cover	E value	Per. Ident
Rhizosphere 1	*Pseudomonas aeruginosa*	2106	2106	99%	0	99.91%
Rhizosphere 2	*E. coli*	2126	14714	100%	0	100.00%
Rhizosphere 3	*Bacillus subtilis*	2113	14624	99%	0	99.91%
Rhizosphere 4	*Enterobacter aerogenes*	2126	14714	100%	0	100.00%
Rhizosphere 5	*Serratia marcescens*	2104	2104	99%	0	99.91%

Molecular analysis from this study indicated that the isolates corresponded to *Bacillus subtilis*, *Pseudomonas aeruginosa, Serratia marcescens, E. coli*, and *Enterobacter aerogenes* with high confidence (over 99% sequence similarity). This molecular validation is crucial for confirming the accuracy of preliminary identifications based on cultural and biochemical tests (Sibley and Peirano et al., 2012).

3.3. Screening Output of Plant Growth Promoting Characteristics of Bacterial Isolates

Of all the bacterial isolates, only *Bacillus subtilis* gave a positive result to the phosphate solubilisation test, indole acetic acid production, ammonia production test, nitrogen fixing test, and show antimicrobial activities while other isolates gave negative results to either one or two PGPR test (Table 3).

Table 3: *In vitro* screening for PGP activities

Bacterial isolates	Ammonia production	Indole acetic acid production	Nitrogen fixation	Phosphate solubilization	Potential antimicrobial activities
Klebsiella species	+	+	-	-	-
Pseudomonas aeruginosa	-	+	-	-	-
E. coli	+	-	-	-	-
Bacillus subtilis	+	+	+	+	+
Enterobacter aerogenes	+	-	+	-	-
Serratia marcescens	+	+	-	-	-
Alcaligenes faecalis	+	+	-	-	-

Key: + (Present/Positive) - (Absent/ Negative)

Among the isolates, *Bacillus subtilis* demonstrated the most significant PGP traits (Table 3). It tested positive for nitrogen fixation, phosphate solubilization, IAA production, and ammonia production, which are crucial for plant health and growth. This findings supported Jabborova and Enakiev et al. (2021) research which previously shows that *Bacillus subtilis* demonstrated plant growth-promoting characteristics by testing positive to siderophore, IAA and caused P-solubilization. In contrast, other isolates like *Klebsiella sp.,*

Pseudomonas aeruginosa, and E. coli showed variable results across these tests, with some lacking in essential PGP traits. This highlights *Bacillus subtilis* as a particularly effective agent for enhancing plant growth in rhizosphere applications (Hashem and Tabassum et al., 2019).

3.4. Cultural Morphology of *Fusarium oxysporum*

Fusarium oxysporum was identified using colony (macroscopic) microscopic and morphology characteristics according to the methods of Shobha and Kumudini (2012). On potato dextrose agar, the fungal colony displayed a white, cotton-like texture with a purple color on the underside. Under the microscope, *Fusarium oxysporum* showed dark, septate hyphae, along with spherical, slightly curved, unicellular, and hyaline microconidia (Table 4).

Table 4: Characterization of *Fusarium oxysporum* isolates obtained from tomato rhizosphere

Parameter	Observation
Cultural Morphology	
Colour on agar plate	White cottony mycelium on plates
Colour of reverse side of culture plate	Dark to purple
Microscopic characteristics	
Nature of hyphae	Septate
Type of Spore	Macroconidia
Conidia structure/attachment	
Rhizoids	Absent
Spore colour	Pink
Appearance of special structure	Spores were oval to ellipsoid/ kidney shaped, oval tapering and septate in three cells whereas chlamydospores formed in chain
Division	Ascomycota
Class of fungi	Ascomycetes
Possible Identity	*Fusarium oxysporum*

3.5. In Vitro Antagonistic Potential and Inhibition rate of *Bacillus* and *Trichoderma* against *Fusarium oxysporum*

Generally, the line chart provides a visual representation of average growth diameter (mm) of fungal mycelial in the treatment groups (*Bacillus* and *Trichoderma*) and control (*Fusarium* only) at 0, 24, 48, 72 and 92 hours respectively. It was observed that the *Bacillus* isolates exhibited antagonistic effects against *F. oxysporum*. The antifungal properties of these *Bacillus* isolates are illustrated in Figure 2. Table 5 showed the rates of inhibition, offering insights into the extent to which *Fusarium oxysporum* is inhibited in the presence and absence of the treatment groups. The *in vitro* antagonistic tests against *Fusarium oxysporum* revealed that *Bacillus* sp. exhibited a notable degree of antifungal activity. *Bacillus isolates* maintained a consistent inhibition rate, peaking at 46.43% at 48 hours. This contrasts with *Trichoderma* sp., which had lower and less stable inhibition rates. The control group, which included *Fusarium* alone, showed a negative inhibition rate at later time points, reflecting uninhibited growth of the pathogen (Figure 2 and Table 5). *Bacillus* species are some of the most extensively studied biological control agents, known for their antagonistic and competitive abilities within the rhizosphere (Etesami and Jeong et al., 2023). This study provides compelling evidence for the antagonistic potential of *Bacillus* spp., isolated from the rhizosphere of *Talinum fruticosum*, against the pathogenic fungus *Fusarium oxysporum*.

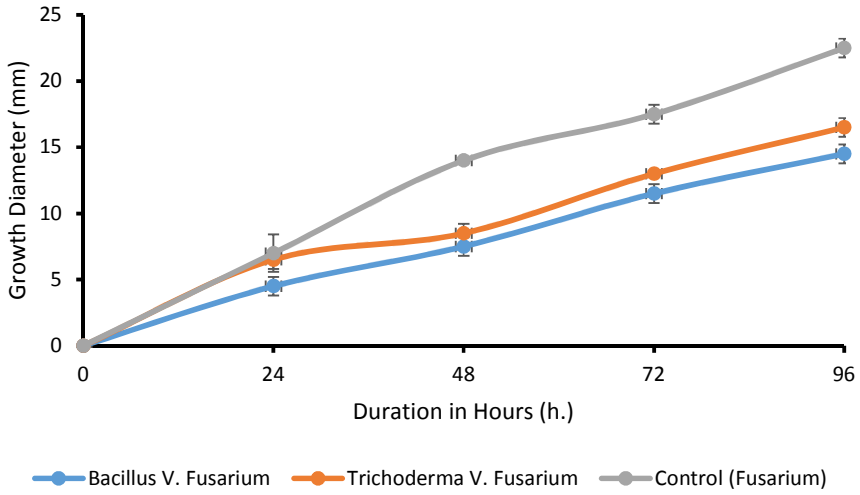

Figure 2: *In vitro* antagonistic potential of *Bacillus* and *Trichoderma* against *Fusarium oxysporum*

The efficacy of *Bacillus* spp. in inhibiting the growth of *F. oxysporum* has been explored by several researchers (Yang and Zhang et al., 2024). Xu and Wang et al. (2020) reported its efficacy in reducing the incidence of *Fusarium* wilt in watermelon while Jangir and Pathak et al. (2018) observed about 36% reduction in wilt disease incidence in tomato plant under greenhouse conditions using *Bacillus* spp . isolated from tomato rhizosphere.

Table 5: Inhibition rate of *Bacillus* and *Trichoderma* against *Fusarium oxysporum*

Time (hours)	*Bacillus* vs *Fusarium* (%)	*Trichoderma* vs *Fusarium* (%)	Control (%)
0	0	0	0
24	35.71	7.14	54.1
48	46.43	39.29	7.8
72	34.29	25.71	-14.8
96	35.56	26.67	-47.5

The significant inhibition observed in this study can be ascribed to antimicrobial compound productions, which are well-documented mechanisms of biocontrol employed by *Bacillus* species (Khan and Maymon et al., 2017). *Bacillus* spp. are known to produce a range of antimicrobial substances, including lipopeptides (LP) like surfactin, iturin, and fengycin , which can inhibit fungal growth by disrupting cell membranes (Wang and Qiu et al., 2022). While the *in vitro* results are promising, the effectiveness of these *Bacillus* isolates under field conditions needs to be evaluated.

4. CONCLUSION

This study underscores the potential of *Bacillus* spp. isolated from *Talinum fruticosum* as effective biocontrol agents against *Fusarium oxysporum.* The findings contribute to the broader understanding of PGPR and their role in sustainable agriculture, offering a promising alternative to chemical-based disease control methods. Further research and field trials will be crucial in translating these *in vitro* findings into practical agricultural solutions.

5. ACKNOWLEDGMENT

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6. CONFLICT OF INTEREST

There is no conflict of interest associated with this work.

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