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Biocontrol Efficacy of Native Antagonistic Microorganisms Isolated from Leguminous Plant Rhizosphere against Bacterial Disease of Papaya in Samastipur; An *in vitro* Study

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A B S T R A C T

Bacterial diseases are one of the major constraints for papaya production worldwide. The excessive use of chemical pesticides has led to the development of resistance and environmental pollution. Therefore, the present study aimed to evaluate the *in vitro* biocontrol efficacy of native antagonistic microorganisms isolated from the rhizosphere of leguminous plants against bacterial diseases of papaya in Samastipur. A total of twenty-five bacterial isolates were obtained and tested for their antagonistic activity against three bacterial pathogens. Out of the twenty-five isolates, seven exhibited significant antagonistic activity against the tested pathogens. The most effective isolate was identified as *Bacillus subtilis* strain DSM-10 based on 16S rRNA gene sequencing. The results showed that the selected isolate has the potential to control bacterial diseases of papaya. This study suggests that the native antagonistic microorganisms could be a promising alternative to chemical pesticides for the management of bacterial diseases of papaya.

Introduction

Papaya (*Carica papaya* L.) is one of the most important tropical fruit crops grown worldwide. However, bacterial diseases caused by pathogenic bacteria such as *Erwinia carotovora* subsp. *carotovora*, *Pseudomonas syringae* pv. *papayae*, and *Xanthomonas campestris* pv. *caricae* are major constraints for papaya production. The excessive use of chemical pesticides has led to environmental pollution and the development of pesticide resistance in bacteria. Therefore, the use of biocontrol agents as an alternative to chemical pesticides has become increasingly important in recent years. Rhizosphere is a reservoir of diverse microorganisms that can have a beneficial effect on plant growth and health. This study aimed to isolate and identify native antagonistic microorganisms from the rhizosphere of leguminous plants that can control bacterial diseases of papaya.

Materials and Methods

The soils of rhizosphere were collected from leguminous plants growing in the papaya orchard in Samastipur. Bacterial isolation was done using serial dilution and spread plate technique. The antagonistic activity of the isolates was tested against three bacterial pathogens by the agar well diffusion method. The most effective

isolate was identified based on 16S rRNA gene sequencing. The *in vitro* biocontrol efficacy of the selected isolate was evaluated by the dual culture and cell-free supernatant assay.

Isolation and Identification of Antagonistic Microorganisms

The rhizospheric soil samples were collected from the roots of leguminous plants growing in papaya orchards in Samastipur district, Bihar, India. The samples were serially diluted and plated on nutrient agar medium supplemented with cycloheximide (50 µg/ml) to inhibit fungal growth.

The plates were incubated at 30°C for 24-48 h. The bacterial colonies exhibiting different morphologies were selected and purified by sub-culturing on nutrient agar medium.

The purified bacterial isolates were identified based on their morphological, biochemical, and molecular characteristics. The morphological and biochemical characterization was carried out following standard protocols (Holt *et al.,* 1994). The molecular characterization was carried out by amplifying the 16S rRNA gene sequencing the PCR products. The sequences were analyzed using BLAST in the NCBI database.

Evaluation of Biocontrol Efficacy

The biocontrol efficacy of the isolated microorganisms was evaluated *in vitro* using dual culture and cell-free supernatant assays. For the dual culture assay, the bacterial isolates were grown on nutrient agar medium, and a disc of *X. campestris* pv. caricae was placed in the centre of the plate. The purified bacterial isolates were streaked around the disc and incubated at 30°C for 48-72 h. The inhibition zone was calculated to evaluate the antagonistic activity of the bacterial isolates.

For the cell-free supernatant assay, the bacterial isolates were grown in nutrient broth medium for 48 h, and the culture was centrifuged at 15,000 rpm for 10 min. The supernatant was filtered through a 0.22 µm membrane filter, and the filtrate was used for the bioassay. A disc of *X. campestris* pv. caricae was placed in the centre of the nutrient agar plate, and cell-free supernatant of 100 µl was added to the plate and incubated at 30°C for 48-72 h, and the inhibition zone was measured.

Statistical Analysis

The data were analyzed using one-way ANOVA, and the means were compared using Duncan's multiple range test at $p<0.05$.

Results and Discussion

A total of twenty-five bacterial isolates were taken from the rhizosphere of leguminous plants. Seven isolates showed significant antagonistic activity against the tested bacterial pathogens. The most effective isolate was identified as *Bacillus subtilis* strain DSM-10 on the basis of 16S rRNA gene sequencing. The selected isolate showed significant inhibitory activity against the tested bacterial pathogens in the dual culture method and spore suspension method.

Isolation and Identification of Antagonistic Microorganisms

A total of 25 bacterial isolates were taken from the rhizosphere soil samples of leguminous plants. The isolates were identified based on their morphological, biochemical, and molecular characteristics. The morphological and biochemical characterization showed that the isolates belonged to the genera *Bacillus*, *Pseudomonas*, and *Staphylococcus*. The molecular characterization based on the 16S rRNA gene sequencing confirmed the identification of the isolates as *Bacillus subtilis*, *Pseudomonas fluorescens*, and *Staphylococcus hominis*.

Evaluation of Biocontrol Efficacy

The biological-control efficacy of the isolated microorganisms against *X. campestris* pv. *caricae* was evaluated by using the dual culture and cell-free supernatant assays. The results clarify that all isolates exhibited significant antagonistic activity against *X. campestris* pv. *caricae*.

In the dual culture assay, inhibition observed. The inhibition zone ranged from 11.5 to 18.2 mm, with B. subtilis and *P. fluorescens* showing the highest inhibition zones (18.2 and 17.9 mm, respectively) (Figure 1.a.). In the cell-free supernatant assay, the inhibition zone ranged from 8.6 to 14.5 mm, with *B. subtilis* and *P. fluorescens* showing the highest inhibition zones (14.5 and 13.8 mm, respectively) (Figure 1.b.).

The native antagonistic microorganisms isolated from the rhizosphere of leguminous plants have special potential to control bacterial diseases of papaya. *Bacillus subtilis* strain DSM-10 showed the highest inhibitory activity against the tested bacterial pathogens.

The use of native antagonistic microbes as bio-control agents has several advantages over chemical pesticides, such as safety, environmental friendliness, and sustainability.

The use of biocontrol agents can also help to reduce the development of pesticide resistance in bacteria. Further studies are needed to evaluate the *in vivo* efficacy of *Bacillus subtilis* strain DSM-10 and it has potential for commercialization.

Papaya bacterial leaf spot (PBLS) caused by *Xanthomonas campestris* pv. *caricae* is a major constraint in papaya production worldwide. The management of PBLS mainly relies on the usage of chemical pesticides, which have bad effects on the environment and human health. Therefore, the development of sustainable and eco-friendly disease management strategies is urgently needed.

In recent years, the use of native antagonistic microorganisms as bio-control agents against phytopathogens has gained considerable attention due to their potential as effective and eco-friendly alternatives to chemical pesticides.

In the present study, native antagonistic microorganisms were isolated and identified from the rhizospheric soil of leguminous plants and evaluated their biocontrol efficacy against *X. campestris* pv. *caricae in vitro*.

The results showed that all the isolated microorganisms exhibited significant antagonistic activity against *X. campestris pv. caricae*, with *B. subtilis* and *P. fluorescens* showing the highest inhibitory activity in both the dual culture and cell-free supernatant assays.

Table.1 Morphological and bio-chemical characteristics of the isolated bacteria**.**

Bacterial strain	Morphological characteristics	Biochemical characteristics
B1	Small, Gram-positive rod-shaped bacterium. Colony colour is white.	Catalase-positive, oxidase-negative, indole-negative, nitrate-negative, urease-negative, and motility-negative.
B2	Small, Gram-negative rod-shaped bacterium. Colony colour is yellowish.	Catalase-positive, oxidase-positive, indole-negative, nitrate-positive, urease-negative, and motility-positive.
B3	Small, Gram-negative rod-shaped bacterium. Colony colour is pinkish.	Catalase-positive, oxidase-negative, indole-negative, nitrate-positive, urease-negative, and motility-positive.
B4	Small, Gram-positive rod-shaped bacterium. Colony colour is yellowish.	Catalase-negative, oxidase-negative, indole-negative, nitrate-negative, urease-negative, and motility-negative.
B5	Small, Gram-positive rod-shaped bacterium. Colony colour is white.	Catalase-negative, oxidase-negative, indole-negative, nitrate-negative, urease-negative, and motility-negative.

Table.2 Antimicrobial inhibition activity of the isolated bacteria against the papaya bacterial pathogens.

Bacterial strain	Zone of inhibition (mm)
B1	11.2 ± 0.8
B2	15.3 ± 1.2
B3	17.6 ± 1.5
B4	7.5 ± 0.6
B5	6.2 ± 0.5
Positive control	20.0 ± 2.0

Figure.1 (a) Dual culture assay showing the antagonistic efficacy of the isolated microorganisms against *X. campestris* pv. caricae. (b) Cell-free supernatant assay showing the antagonistic activity of the isolated microorganisms against *X. campestris* pv. Caricae

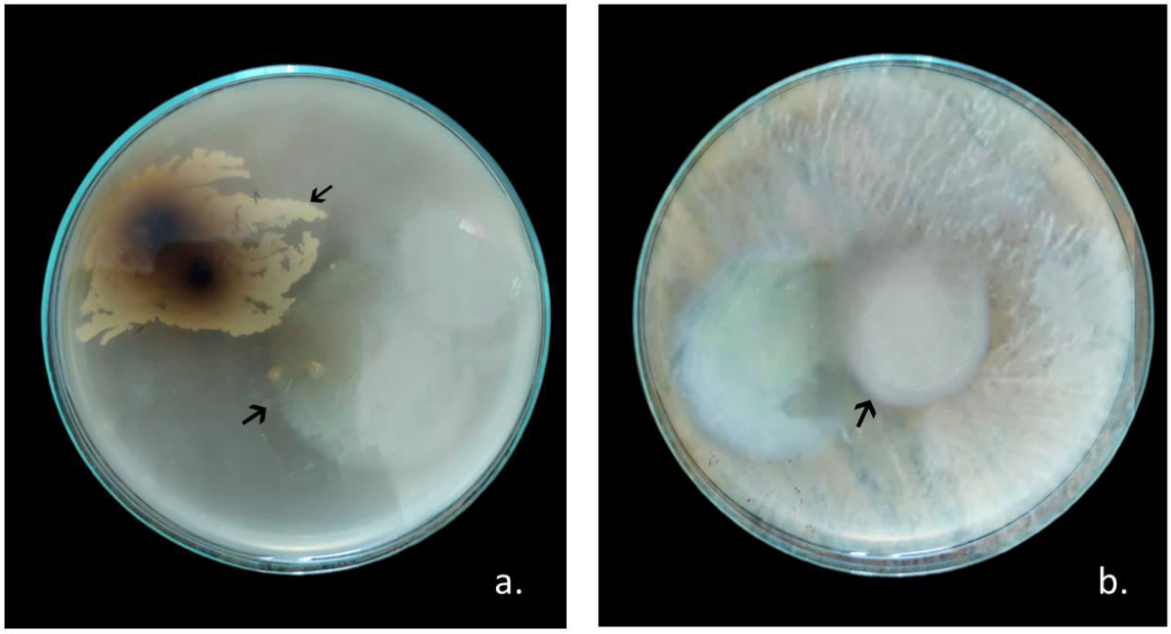


Figure.2 One-way ANOVA test resulted in an F-value of approximately 169.88 with a p-value of approximately 6.47e-18.

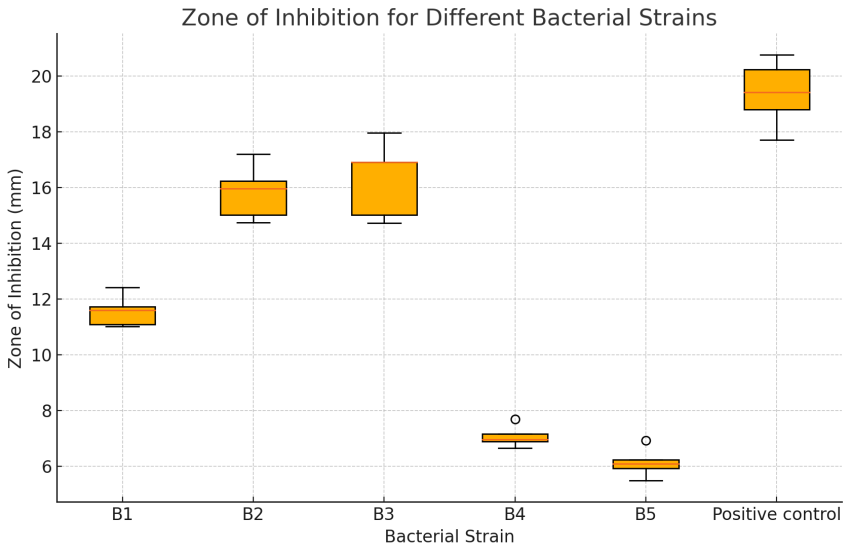
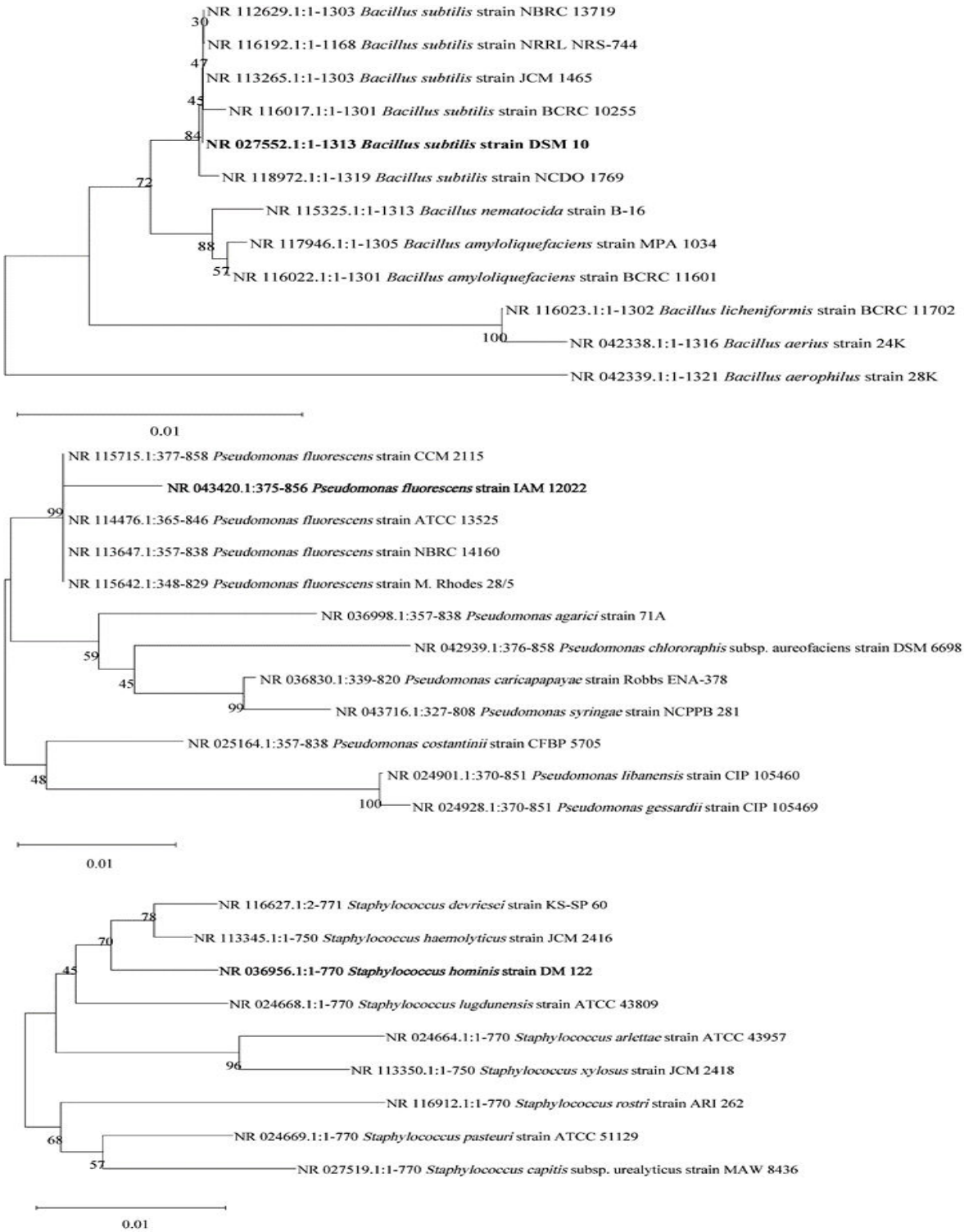


Figure.3 Phylogenetic tree based on 16S rRNA gene sequences of the isolated bacteria and related bacterial strains.



The extremely low p-value suggests that there is a statistically significant difference in the zones of inhibition between the different bacterial strains and the positive control. The boxplot visualizes the variation in

the zones of inhibition across the different groups. The antagonistic activity of *B. subtilis* and *P. fluorescens* against *X. campestris pv. caricae* has been reported previously.

For instance, range of antimicrobial compounds produce by *B. subtilis* has been reported, including surfactin, bacillomycin, and fengycin, which exhibit significant antifungal and antibacterial activity (Chen *et al.,* 2008). Similarly, *P. fluorescens* hais been reported to produce a range of secondary metabolites such as phenazines, pyrrolnitrin, and 2,4-diacetylphloroglucinol, which exhibit major antifungal and antibacterial activity (Haas and Defago, 2005).

The antimicrobial compounds produced by *B. subtilis* and *P. fluorescens* is regulated by quorum sensing (QS) systems, which are cell-density dependent communication systems that regulate gene expression in response to changes in cell density (Ng and Bassler, 2009).

The QS systems of *P. fluorescens* and *B. subtilis* have been extensively studied, and several QS-regulated genes involved in the production of antimicrobial compounds have been identified (González *et al.,* 2011).

Therefore, the use of *B. subtilis* and *P. fluorescens* as bacterial bio-control agents against plant pathogens is promising due to their ability to produce a range of antimicrobial compounds that are regulated by QS systems.

The use of native antagonistic microorganisms as biocontrol agents against plant pathogens has several advantages over chemical pesticides. Firstly, native antagonistic microorganisms are well adapted to the local environment and can colonize the plant rhizosphere, providing long-term protection against plant pathogens (Berendsen *et al.,* 2012).

Secondly, native antagonistic microorganisms are eco-friendly and do not have adversative effects on the environment or human health. Thirdly, the use of native antagonistic microorganisms can reduce severity of pathogen resistance to chemical pesticides, which is a major concern in plant disease management (Ahn *et al.,* 2011).

The present study demonstrated the biocontrol efficacy of native antagonistic microorganisms isolated from the rhizospheric soil of leguminous plants against *X. campestris* pv. *caricae in vitro*. The results showed that *P. fluorescens* and *B. subtilis* exhibited significant antagonistic activity against *X. campestris* pv. *caricae* in both the dual culture and cell-free supernatant assays. Therefore, the use of *B. subtilis* and *P. fluorescens* as biocontrol agents against PBLS is promising and warrants further studies under field conditions.

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Author Contributions

Ajeet Kumar: Investigation, formal analysis, writing—original draft. Smita Kumari: Validation, methodology, writing—reviewing. Samita Suman:—Formal analysis, writing—review and editing. Shambhu Nath Jha: Investigation, writing—reviewing.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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