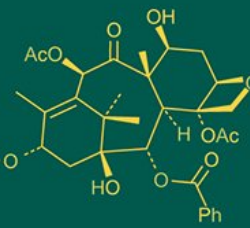
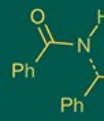
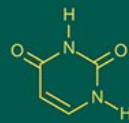


International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
ISSN Online: 2617-4707
NAAS Rating (2026): 5.29
IJABR 2026; SP-10(7): 315-320
www.biochemjournal.com
Received: 25-04-2026
Accepted: 27-05-2026

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Evaluation of indigenous *Bacillus subtilis* and *Pseudomonas fluorescens* for the sustainable biological management of new emerging pomegranate wilt caused by *Ceratocystis fimbriata*

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DOI: <https://www.doi.org/10.33545/26174693.2026.v10.i7Sd.8987>

Abstract

Pomegranate wilt caused by *Ceratocystis fimbriata* has recently emerged as a serious threat to sustainable pomegranate production, leading to substantial economic losses and posing significant challenges to disease management. The present investigation was undertaken to evaluate the biocontrol potential of indigenous isolates of *Bacillus subtilis* and *Pseudomonas fluorescens* for the sustainable biological management of this emerging disease. A total of six indigenous isolates each of *B. subtilis* (BS1–BS6) and *P. fluorescens* (PS1–PS6) were evaluated under *in vitro* conditions using the dual culture technique against *C. fimbriata* isolate PCF-5. Significant differences were observed among the bacterial isolates in their antagonistic activity against the pathogen. Among the *B. subtilis* isolates, BS6 exhibited the highest antagonistic potential by restricting the radial mycelial growth of *C. fimbriata* to 24.23 mm, resulting in 73.07% mycelial inhibition, followed by BS1 (64.33%) and BS3 (60.87%). The lowest inhibition was recorded with BS5 (44.15%). Similarly, among the *P. fluorescens* isolates, PS3 proved to be the most effective, suppressing pathogen growth to 32.33 mm with 64.07% inhibition, followed by PS2 (59.52%) and PS1 (58.30%), whereas PS4 exhibited the lowest inhibition (49.81%). The findings demonstrate considerable variability among indigenous bacterial isolates and highlight BS6 and PS3 as the most promising candidates for biological suppression of *C. fimbriata*. These results provide a scientific basis for the development of eco-friendly and sustainable microbial formulations for integrated management of emerging pomegranate wilt.

Keywords: *Bacillus subtilis*, *Pseudomonas fluorescens*, *Ceratocystis fimbriata*, Pomegranate

Introduction

Pomegranate (*Punica granatum* L.) is an economically important fruit crop cultivated extensively in tropical and subtropical regions because of its high nutritional, medicinal and export value. India ranks first in global pomegranate production, with Maharashtra being the major producing state, followed by Karnataka, Gujarat, Andhra Pradesh and Rajasthan. The fruit is rich in antioxidants, polyphenols, vitamins and minerals, making it highly valued in both domestic and international markets. Besides its commercial significance, pomegranate cultivation provides a reliable source of income for farmers, particularly in the semi-arid regions of India (Jalilop, 2007; Holland *et al.*, 2009)^[13, 15].

Among the major constraints affecting pomegranate production, wilt caused by *Ceratocystis fimbriata* Ellis & Halsted has recently emerged as one of the most destructive diseases. The pathogen causes yellowing, wilting, vascular discoloration, bark splitting, gum exudation, root decay and eventual death of infected plants. Its persistence in soil and spread through infected planting material, contaminated tools, irrigation water, and wounds make disease management difficult, leading to serious economic losses in major pomegranate-growing regions of Maharashtra (Ploetz, 2003; Engelbrecht and Harrington, 2005; Van Wyk *et al.*, 2013)^[6, 11, 22, 25].

Conventional management of vascular wilt relies largely on chemical fungicides, but their effectiveness is often limited because the pathogen colonizes internal vascular tissues. Moreover, excessive fungicide use increases production costs, leaves chemical residues, and

Adversely affects beneficial soil microorganisms. Therefore, sustainable and environmentally safe disease management strategies are increasingly being emphasized (Cook and Baker, 1983; Harman *et al.*, 2004) [2, 5, 10]. Plant growth-promoting rhizobacteria (PGPR) have gained considerable attention as effective biological control agents against soil-borne pathogens. Among them, *Bacillus subtilis* and *Pseudomonas fluorescens* are widely recognized for their ability to suppress plant pathogens through the production of antimicrobial metabolites, siderophores, hydrolytic enzymes, and volatile compounds, while also enhancing plant growth and inducing systemic resistance (Kloepper *et al.*, 2004; Haas and Défago, 2005; Lugtenberg and Kamilova, 2009; Ongena and Jacques, 2008) [9, 17, 19, 20]. Considering the increasing incidence of pomegranate wilt and the need for sustainable disease management, the present investigation was undertaken to isolate and characterize *Ceratocystis fimbriata* from wilt-affected pomegranate plants collected from major pomegranate-growing regions of Maharashtra. The study further evaluated the *in vitro* antagonistic potential of indigenous isolates of *Bacillus subtilis* and *Pseudomonas fluorescens* against the pathogen using the dual culture technique to identify effective biocontrol agents for integrated management of pomegranate wilt.

2. Materials and methods

2.1 Isolation and purification of *Ceratocystis fimbriata*

Ceratocystis fimbriata was isolated from wilt-infected pomegranate root tissues collected from diseased plants at different location in Maharashtra. The infected roots were thoroughly washed under running tap water, cut into approximately 1 cm pieces, surface sterilized with 1% sodium hypochlorite (NaOCl) for 2 min, followed by 70% ethanol and rinsed twice with sterile distilled water. Small tissue bits (1–2 mm) from the advancing margin of infection were aseptically placed on potato dextrose agar (PDA) plates and incubated at 20 ± 1 °C in a BOD incubator. Emerging fungal colonies were purified by the hyphal-tip technique on 2% water agar. Briefly, a dilute spore suspension prepared from a 15-day-old culture was spread uniformly on water agar and after spore germination, individual hyphal tips were transferred to fresh PDA plates. The purified cultures were incubated at 20 ± 1 °C for 15 days and examined for uniform colony growth without sectoring to ensure culture purity before further studies.

2.2 Pathogenicity test, re-isolation and disease assessment of *Ceratocystis fimbriata*

Pathogenicity of *Ceratocystis fimbriata* was confirmed by fulfilling Koch's postulates using six-month-old pomegranate (*Punica granatum* L.) cv. Bhagwa seedlings maintained under greenhouse conditions. A spore suspension was prepared by inoculating a 5 mm mycelial disc from a 15-day-old culture into sterilized potato dextrose broth (PDB) supplemented with streptomycin sulphate and incubated at room temperature for 12 days. The resulting mycelial mat was homogenized and the spore concentration was adjusted to 2.5×10^6 spore's ml⁻¹. A slanting wound (approximately 3 cm deep) was made at the collar region of each seedling using a sterile knife. Each wound was inoculated with 0.2 ml of the spore suspension and a 5 mm mycelial plug, followed by sealing with parafilm as described by Harrington *et al.* (2005) [12]. Control plants were inoculated with sterile distilled water. The inoculated seedlings were maintained under greenhouse conditions and monitored daily for symptom development. Typical wilt symptoms developed approximately 25 days after

inoculation. The pathogen was re-isolated from symptomatic tissues by surface sterilization with 1% sodium hypochlorite, followed by plating on potato dextrose agar (PDA) and incubation at 20 ± 1 °C. The recovered fungus was identified based on its colony morphology and microscopic characteristics, which were identical to those of the original culture, thereby confirming Koch's postulates. Disease incidence was recorded using the following **formula:** Percent Disease Incidence (PDI) = (Number of plants showing wilt symptoms / Total number of plants examined) $\times$ 100 Based on the percent disease incidence, the isolates of *C. fimbriata* were classified into virulence groups according to the scale proposed by Sharma *et al.* (2010) [24].

2.3 Statistical analysis

Radial mycelial growth of *Ceratocystis fimbriata* was recorded at 24-hour intervals until the pathogen completely covered the untreated control plates. Percent inhibition of mycelial growth by the bioagents was calculated using the formula of Arora and Upadhyay (1978) [1, 27]:

$$\text{Percent inhibition (\%)} = \left(\frac{C - T}{C} \right) \times 100$$

Where, C = radial growth of the pathogen in the untreated control (mm) and T = radial growth of the pathogen in the treatment (mm). The experiment was conducted in a Completely Randomized Design (CRD) with seven treatments and three replications. The experimental data were subjected to analysis of variance (ANOVA) using WASP 1.0 statistical software developed by the ICAR–Central Coastal Agricultural Research Institute (ICAR–CCARI), Goa. Treatment means were compared at the 5% level of significance ($P = 0.05$).

3. Result and discussion

3.1 Symptomatology

Pomegranate plants infected with *Ceratocystis fimbriata* exhibited typical wilt symptoms in both above- and below-ground plant parts. Initial symptoms appeared as yellowing and wilting of leaves on one or a few branches, which gradually progressed to the entire canopy. In severely affected plants, rapid wilting, leaf drying and retention of dried leaves and fruits on the branches were commonly observed. Vertical stem cracking, bark splitting and dark brown vascular discoloration beneath the bark were prominent diagnostic symptoms. In many orchards, diseased plants occurred in patches, indicating local spread of the pathogen to neighbouring plants. These observations are in agreement with the findings of Huang *et al.* (2003) [14], Sharma *et al.* (2010), Khosla *et al.* (2011), Xu *et al.* (2011), and Chaudhari *et al.* (2016) [4, 16, 24, 2]. The underground portions of infected plants showed extensive root rot and dark grey to brown discoloration of the vascular tissues, particularly in the collar and root regions. A characteristic alcoholic odour was also evident in severely infected roots. The pathogen survives in infected plant tissues and soil through resistant propagules and is disseminated mainly through infected planting material, contaminated soil and irrigation water, root contact, insect vectors and contaminated pruning tools. These observations corroborate earlier reports by Somasekhara *et al.* (2009), Sharma *et al.* (2010), Khosla *et al.* (2011), and Bhardwaj *et al.* (2013) [3, 16, 24, 26].

3.2 Isolation and identification of *Ceratocystis fimbriata*

3.1.1 Isolation and coding of isolates

Ceratocystis fimbriata was isolated from root tissues of wilt-affected pomegranate plants collected from major pomegranate-growing districts of Maharashtra. Following

surface sterilization, tissue segments cultured on potato dextrose agar (PDA) developed characteristic whitish-grey fungal colonies within 24 h of incubation at $20 \pm 1^\circ\text{C}$, indicating successful isolation of the pathogen. The isolation procedure and colony characteristics were consistent with those described by Sharma *et al.* (2010) [24]. A total of 25 isolates were obtained and designated as PCF (Pomegranate *Ceratocystis fimbriata*) isolates. All isolates were purified, maintained on PDA, and utilized for subsequent studies on pathogenic variability and biological characterization.

3.1.2 Identification of *Ceratocystis fimbriata*

The pathogen was identified based on its cultural and microscopic characteristics using 15-day-old PDA cultures in Plate 1. Colonies were initially whitish-grey and gradually turned grey to brown with profuse, septate, branched, smooth-walled mycelia and circular to irregular colony margins. Mature cultures produced abundant black, globose perithecia with elongated necks bearing characteristic fimbriate ostioles. Cream-coloured mucilaginous droplets containing hat-shaped ascospores were observed exuding from the perithecial necks, a distinguishing feature of *C. fimbriata*, as previously described by Upadhyay (1981) and Baker *et al.* (2003) [2, 27]. Microscopic examination revealed flask-shaped primary phialides producing hyaline, aseptate, cylindrical endoconidia, whereas secondary phialides produced hyaline, barrel-shaped endoconidia. Doliform endoconidia were initially hyaline and gradually became pale brown on maturation. Thick-walled, pigmented aleurioconidia were produced singly on simple or branched conidiophores. The combination of these cultural and morphological characteristics confirmed the identity of the fungus as *Ceratocystis fimbriata*, in agreement with the descriptions of Harrington (2000) [11], Roux *et al.* (2007), and Sharma *et al.* (2010) [23, 24].

4. Pathogenic Variability of *Ceratocystis fimbriata*

Pathogenic variability among 25 isolates of *Ceratocystis fimbriata* was assessed on six-month-old pomegranate cv. Bhagwa seedlings under greenhouse conditions. All isolates induced characteristic wilt symptoms, including leaf yellowing, wilting, vascular discoloration, collar rot, and greyish mycelial growth. Symptom initiation varied from 29 to 43 days after inoculation (DAI), followed by complete wilting within 53-69 DAI. The pathogen was successfully re-isolated from infected plants, thereby fulfilling Koch's postulates. Disease incidence ranged from 40 to 100%, demonstrating substantial variation in virulence among the isolates. Isolate PCF-5 was the most virulent, producing the earliest symptom expression (29 DAI) and complete wilting by 53 DAI with 100% disease incidence in Plat 2. Based on pathogenicity, 12 isolates were classified as highly pathogenic (>70% disease incidence), nine isolates as strongly pathogenic (51–70%), and four isolates as moderately pathogenic (21–50%), whereas no non-pathogenic or weakly pathogenic isolates were detected. The observed variability in disease incidence and symptom development indicates marked differences in aggressiveness among *C. fimbriata* isolates, highlighting the importance of pathogen characterization for epidemiological studies, resistance screening, and the development of effective wilt management strategies.

In vitro Antagonistic Activity of *Bacillus subtilis* Isolates

Significant differences were observed among the *Bacillus subtilis* isolates in their antagonistic activity against *Ceratocystis fimbriata* (PCF-5) (Table 1; Plate 3; Fig. 1). Among the six isolates, BS6 was the most effective, restricting mycelial growth to 24.23 mm with 73.07% inhibition over the control. This was followed by BS1 (32.10 mm; 64.33% inhibition) and BS3 (35.22 mm; 60.87% inhibition). Moderate inhibition was recorded with BS4 (59.39%) and BS2 (55.89%), whereas BS5 was the least effective, recording 50.27 mm radial growth with 44.15% inhibition. The untreated control exhibited complete mycelial growth (90.00 mm). The differences among treatments were statistically significant ($\text{SE (m)} \pm = 0.34$; $\text{CD at } P = 0.01 = 1.42$).

Table 1: *In vitro* efficacy of different *Bacillus subtilis* against *Ceratocystis fimbriata* (PCF-5).

Tr No.	Treatment	Average radial mycelial growth (mm)	Per cent Inhibition (%)
T1	BS 1	32.10	64.33
T2	BS 2	39.70	55.89
T3	BS 3	35.22	60.87
T4	BS 4	36.55	59.39
T5	BS 5	50.27	44.15
T6	BS 6	24.23	73.07
T7	Control	90.00	0.00
	F Test	Sig.	-
	SE(m±)	0.34	-
	CD@(0.01%)	1.42	-

4.10.4.3 *in vitro* Antagonistic Activity of *Pseudomonas fluorescens* Isolates

The *Pseudomonas fluorescens* isolates also exhibited significant antagonistic activity against *C. fimbriata* (Table 2; Plate 4; Fig. 2). Among the isolates, PS3 showed the highest inhibition, restricting pathogen growth to 32.33 mm with 64.07% inhibition, followed by PS2 (59.52%) and PS1 (58.30%). Moderate inhibition was observed with PS5 (57.56%) and PS6 (53.19%), whereas PS4 was the least effective, recording 45.17 mm radial growth and 49.81% inhibition. The untreated control showed maximum mycelial growth (90.00 mm). Statistical analysis revealed significant differences among the treatments ($\text{SE(m)} \pm = 0.49$; $\text{CD at } P = 0.01 = 2.08$).

Table 2: *In vitro* efficacy of different *Pseudomonas fluorescens* against *Ceratocystis fimbriata*

Tr No.	Treatment	Average radial mycelial growth (mm)	Percent Inhibition (%)
T1	PS 1	37.53	58.30
T2	PS 2	36.43	59.52
T3	PS 3	32.33	64.07
T4	PS 4	45.17	49.81
T5	PS 5	38.20	57.56
T6	PS 6	42.13	53.19
T7	Control	90.00	0.00
	F Test	Sig.	-
	SE(m±)	0.49	-
	CD@(0.01%)	2.08	-

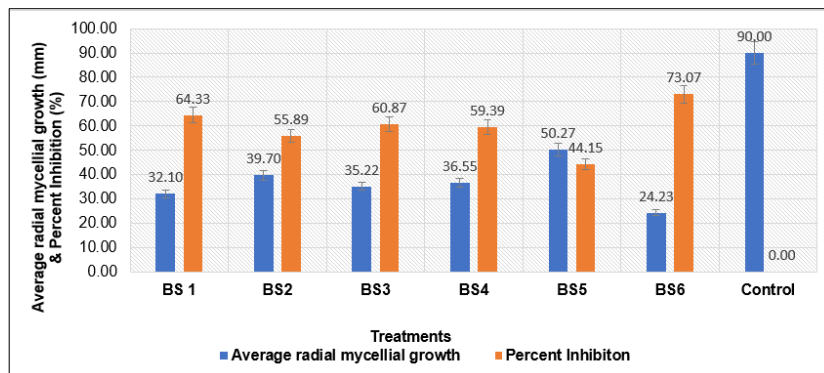


Fig 1: In-vitro antagonistic activity of *bacillus subtilis* against *ceratocystis fimbriata* (pcf-5)

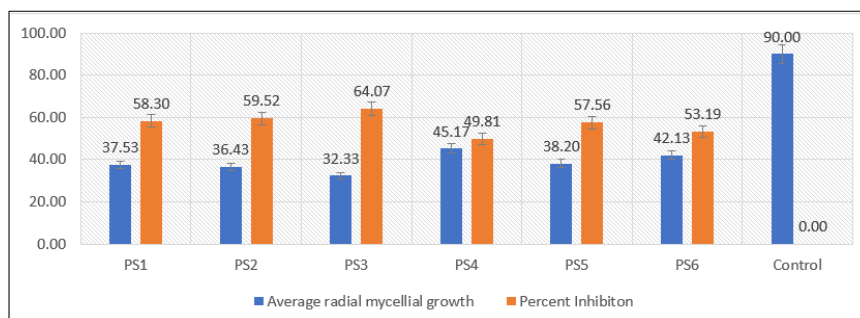


Fig 2: In-vitro antagonistic activity of *pseudomonas fluorescens* against *ceratocystis fimbriata* (pcf-5)

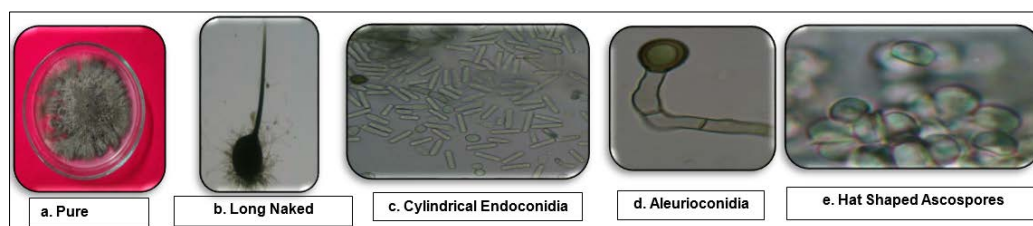


Plate 1: Identification and morphology of *Ceratocystis fimbriata* isolates associated with

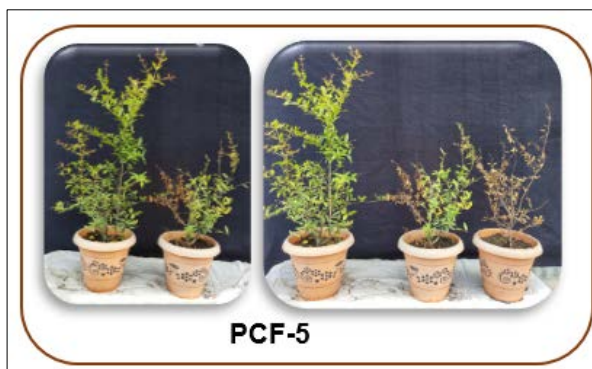


Plate 2: Highly pathogenic isolates of *ceratocystis fimbriata* based on pathogenicity symptoms

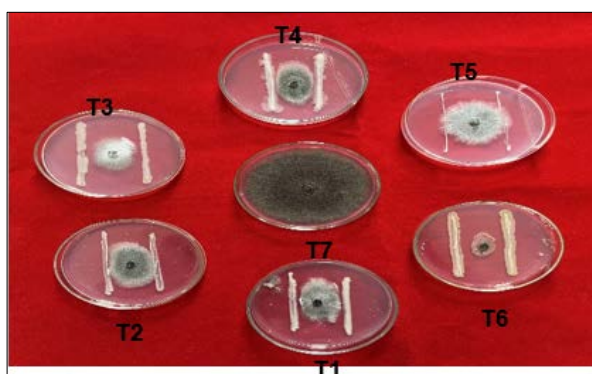


Plate 3: In vitro efficacy of different *Bacillus subtilis* against *Ceratocystis fimbriata* (PCF-5)

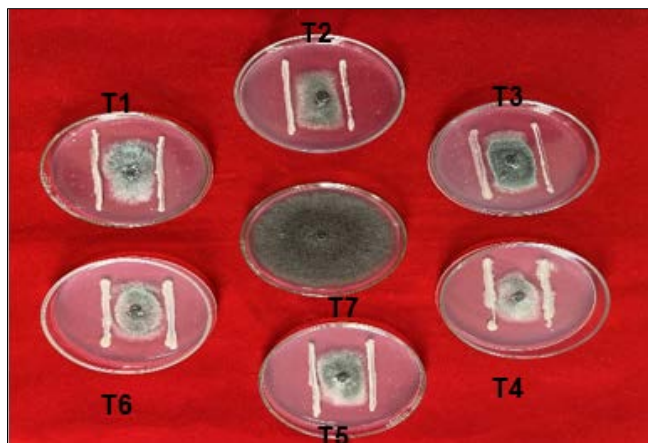


Plate 4: *In vitro* efficacy of different *pseudomonas fluorescens* against *ceratocystis fimbriata* (pcf-5)

Conclusion

The present study confirmed *Ceratocystis fimbriata* as the causal agent of pomegranate wilt based on symptomatology, cultural and morphological characteristics and pathogenicity tests. Considerable pathogenic variability was observed among the 25 isolates, with disease incidence ranging from 40 to 100%. Isolate PCF-5 was identified as the most virulent, exhibiting the earliest symptom development and highest disease incidence, indicating significant differences in aggressiveness among the pathogen population.

Among the biological control agents evaluated, *Bacillus subtilis* isolate BS6 exhibited the highest antagonistic activity against *C. fimbriata*, with 73.07% inhibition of mycelial growth, followed by BS1 and BS3. Similarly, *Pseudomonas fluorescens* isolate PS3 showed the greatest inhibition (64.07%), followed by PS2 and PS1. Overall, *B. subtilis* isolates were more effective than *P. fluorescens* isolates under *in vitro* conditions. These findings suggest that the superior antagonistic isolates, particularly BS6 and PS3, have significant potential for the biological management of pomegranate wilt and warrant further evaluation under greenhouse and field conditions for their integration into sustainable disease management strategies.

Acknowledgment

Authors acknowledge the Department of Plant Pathology, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra, India for providing the laboratory facilities required for conducting this research.

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