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ANTAGONISTIC POTENTIAL OF *TRICHODERMA* SPP. AGAINST *CERATOCYSTIS FIMBRIATA* CAUSING WILT OF POMEGRANATE

Petkar G.V.[1]*, R. W. Ingle[2], V. S. Wargane[1], M. V. Totawar[1], S. B. Brahmankar[1], S. T. Ingle[1] and N. S. Satpute[3]

[1]Department of Plant Pathology, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola-444104, Maharashtra, India
[2]Department of Plant Pathology Agri. College of Nagpur, Maharashtra, India
[3]Department of Agril. Entomology, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola-444104, Maharashtra, India
*Corresponding author E-mail: shamkpetkar88@gmail.com
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ABSTRACT

Wilt of pomegranate caused by *Ceratocystis fimbriata* is a major constraint to pomegranate production in Maharashtra, leading to severe yield losses and orchard decline. The present investigation was undertaken to study the occurrence of pomegranate wilt across major pomegranate-growing regions of Maharashtra and to evaluate the antagonistic potential of native *Trichoderma* spp. against the pathogen under *in vitro* conditions. Wilt-affected pomegranate samples were collected from different locations and the causal organism was isolated through tissue isolation techniques. The pathogenicity of the isolated *C. fimbriata* was confirmed by standard pathogenicity tests, thereby fulfilling Koch's postulates. An in vitro evaluation of selected *Trichoderma* species was conducted using the dual culture technique to assess their antagonistic activity against *C. fimbriata*. Six *Trichoderma* species, namely *T. harzianum*, *T. longibrachiatum*, *T. koningii*, *T. asperelloides*, *T. asperellum* and *T. hamatum*, were screened for their inhibitory effect on mycelial growth of the pathogen. All tested *Trichoderma* isolates exhibited varying degrees of antagonism against *C. fimbriata*. Among them, *Trichoderma hamatum* recorded the maximum mycelial growth inhibition (76.62%), indicating strong antagonistic potential, whereas *T. harzianum* showed the least inhibition (58.51%). The remaining species demonstrated moderate levels of suppression of the pathogen. The results of the present study clearly demonstrate the effectiveness of certain native *Trichoderma* strains in inhibiting the growth of *C. fimbriata* under laboratory conditions. These findings highlight the potential of region-specific *Trichoderma* isolates as eco-friendly and sustainable components of integrated disease management strategies for pomegranate wilt.

Keywords: *Ceratocystis fimbriata, Trichoderma* spp. Pomegranate wilt, dual culture, biological control.

Introduction

Pomegranate (*Punica granatum* L.) is an economically important fruit crop extensively cultivated in the arid and semi-arid regions of India due to its high adaptability, nutritional value, medicinal properties and export potential (Jalikop, 2007; Holland *et al*., 2009). India is one of the leading producers of pomegranate, with Maharashtra contributing a major share to national production. However, commercial cultivation of pomegranate is severely constrained by several biotic stresses, among which wilt disease is one of the most destructive, leading to significant yield losses and orchard mortality (Sharma *et al*., 2013).

Wilt of pomegranate is predominantly caused by soil-borne fungal pathogens, particularly *Ceratocystis fimbriata*, which infects the vascular tissues and disrupts water and nutrient transport (Chaudhari *et al*., 2011). The disease is characterized by progressive leaf yellowing, sudden wilting, vascular discoloration, stem staining, root decay and eventual plant death. Disease severity is influenced by environmental factors such as high soil moisture, elevated temperatures and continuous monocropping, which favor pathogen

survival and spread (Jadhav and Sharma, 2009). Once established, the pathogen persists in soil and plant debris for extended periods, making its management difficult.

Conventional management of pomegranate wilt relies largely on chemical fungicides; however, their effectiveness is limited due to poor soil mobility, environmental pollution, disruption of beneficial soil microflora, and the risk of development of pathogen resistance (Pal and Gardener, 2006). These constraints have necessitated the development of sustainable and eco-friendly alternatives for disease management.

Biological control using antagonistic fungi, particularly species of *Trichoderma*, has gained considerable attention as an effective strategy for managing soil-borne plant pathogens (Harman *et al*., 2004). *Trichoderma* spp. suppresses pathogens through multiple mechanisms, including mycoparasitism, antibiosis, competition for nutrients and space and induction of systemic resistance in host plants (Howell, 2003; Vinale *et al*., 2008). However, the efficacy of *Trichoderma* varies among species and strains, emphasizing the importance of region-specific screening (Mukherjee *et al*., 2012).

In view of the above, the present investigation was undertaken to isolate *Ceratocystis fimbriata* from wilt-affected pomegranate samples collected from major pomegranate-growing regions of Maharashtra and to confirm its pathogenicity. The study also aimed to document disease symptomatology and morphological characteristics of the pathogen and to evaluate the *in vitro* antagonistic potential of different *Trichoderma* species against *C. fimbriata* using the dual culture technique, with the objective of identifying effective biocontrol agents for integrated wilt management.

Materials and methods

Isolation of the pathogen

Ceratocystis fimbriata were collected and processed for pathogen isolation. The diseased root samples were initially washed thoroughly under running tap water to remove adhering soil particles and debris. The cleaned roots were then cut into small segments of approximately 1 cm in length and surface sterilized with 1% sodium hypochlorite (NaOCl) solution for 2 minutes to eliminate surface contaminants. Following sterilization, the segments were rinsed briefly in 70% ethanol and subsequently washed twice with sterile distilled water to remove any residual traces of sterilant (Tuite, 1969; Dhingra and Sinclair, 1995).

After surface sterilization, each 1 cm segment was further cut into smaller pieces of about 1 mm in size under aseptic conditions. These tissue bits were aseptically transferred onto sterile potato dextrose agar (PDA) medium in Petri plates. The inoculated plates were incubated in a biological oxygen demand (BOD) incubator at 20 ± 1°C for 24 hours. The emergence of characteristic whitish-grey mycelial growth from the plated tissues indicated successful isolation of the pathogen. The actively growing mycelium was subsequently subcultured onto fresh PDA plates to obtain pure cultures for further morphological and pathogenicity studies (Barnett and Hunter, 1998).

Pathogenicity

Pathogenicity tests were conducted to fulfill Koch's postulates using six-month-old healthy pomegranate seedlings (cv. Bhagva) maintained under greenhouse conditions. A downward slanting incision of approximately 3 cm depth was made at the basal portion of the stem using a sterile scalpel. A spore suspension of *Ceratocystis fimbriata* adjusted to 2.5×10^6 spores ml^{-1} was prepared, and 0.2 ml of the suspension along with a 5 mm mycelial disc from an actively growing culture was aseptically placed at the wound site. The inoculated area was sealed with parafilm following the method described by Harrington *et al*. (2005). Control plants were similarly wounded and treated with sterile distilled water. All plants were maintained under greenhouse conditions and observed regularly. Typical wilt symptoms developed within 29 days of inoculation, while control plants remained symptomless. Re-isolation of the pathogen from infected tissues confirmed pathogenicity.

In vitro efficacy of *Trichoderma* spp. against *Ceratocystis fimbriata*

Six *Trichoderma* species were evaluated for their antagonistic potential against a highly virulent isolate of *Ceratocystis fimbriata* obtained from wilt-affected pomegranate plants collected from major pomegranate-growing regions of Maharashtra. The antagonistic activity of the *Trichoderma* isolates was assessed *in vitro* using the dual culture technique on potato dextrose agar (PDA) medium, along with an untreated pathogen-inoculated control (Dennis and Webster, 1971).

A 5 mm diameter mycelial disc was aseptically cut from the margin of an actively growing culture of *C. fimbriata* and placed at one side of a sterile PDA plate. Similarly, a 5 mm mycelial disc of the respective *Trichoderma* isolate was placed on the opposite side of the same plate. Plates inoculated with *C. fimbriata* alone, without *Trichoderma* inoculation, were

maintained as control. Each treatment was replicated three times.

All inoculated plates were incubated at room temperature for 15 days. The extent of antagonism was assessed by measuring the radial growth of *C. fimbriata* in both treatment and control plates. The percent inhibition of mycelial growth over control was calculated using the formula proposed by Vincent (1927). Details regarding the source and origin of the *Trichoderma* isolates used in the study a pure culture of biocontrol agents viz. *T. harzianum*, *T. longibrachiatum*, *T. koningii*, *T. asperelloides*, *T. asperellum* and *T. hamatum* obtained from the biocontrol laboratory, Department of plant pathology, Dr. P.D.K.V. Akola, maintained and multiplied on appropriate culture media and used for further studies.

Statistical treatment of data

Observations on linear mycelial growth of the test pathogen and the bioagents were recorded at 24-hour intervals and continued until the untreated control plates were completely covered by the mycelial growth of the test fungus. The percent inhibition of mycelial growth of the pathogen by the respective bioagents over the untreated control was calculated using the formula described by Arora and Upadhyay (1978).

The recorded data were subjected to statistical analysis following a Completely Randomized Design (CRD). Analysis was carried out using WASP-1.0 statistical software developed by the Central Coastal Agricultural Research Institute, Indian Council of Agricultural Research (ICAR-CCARI), Goa, India (ICAR-CCARI, 2024). The antagonistic effects of the six *Trichoderma* species evaluated in the present study are presented in Table 1.

Formula used for percent growth inhibition

Per cent growth inhibition =

$$\frac{\text{Colony growth in control plate} - \text{Colony growth in treated plate}}{\text{Colony growth in control plate}} \times 100$$

Details of the experiment of Bioagents:

Design : CRD

Replication : Three

Treatments: Seven

Table 1 : Treatment details of fungal bioagent *Trichoderma* spp.

Tr No.	*Trichoderma sp.*
T_1	*Trichoderma harzianum*
T_2	*Trichoderma longi*
T_3	*Trichoderma koningi*
T_4	*Trichoderma asperolloid*
T_5	*Trichoderma hamatum*
T_6	*Trichoderma asperellum*
T_7	Control

Results and Discussion

Symptomatology

Wilt incidence in pomegranate orchards was confirmed through above- and below-ground observations. Above-ground symptoms of *Ceratocystis fimbriata* infection initially appeared as yellowing of foliage on one or a few branches, which gradually progressed to wilting of the entire canopy in Fig 1. In some cases, wilting was confined to a few stems for several weeks or months before complete plant collapse, while sudden wilt characterized by rapid senescence of the entire foliage was also observed. Wilted trees often retained dried leaves and fruits on the branches, and disease occurrence was frequently patchy within orchards, indicating plant-to-plant spread. Vertical stem cracking and internal vascular discoloration were common diagnostic features. Similar symptoms have been reported by earlier workers (Huang *et al.*, 2003; Sharma *et al.*, 2010; Khosla *et al.*, 2011; Xu *et al.*, 2011; Chaudhari *et al.*, 2016). Below-ground examination revealed dark greyish-brown vascular streaking, root rot, and a characteristic alcoholic odour produced by the pathogen, confirming wilt infection (Sharma *et al.*, 2010). The pathogen survives in host tissues and soil for extended periods and spreads through infected planting material, irrigation water, root contact, insects, and contaminated tools, as documented earlier (Somasekhara *et al.*, 2009; Bhardwaj *et al.*, 2013).

Isolation and morphology of the *Ceratocystis fimbriata* causing wilt of pomegranate

Ceratocystis fimbriata, the causal agent of pomegranate wilt, was isolated from infected root and stem tissues collected from major pomegranate-growing regions of Maharashtra using the tissue isolation technique. A total of twenty-five isolates were obtained, of which one highly virulent isolate (PCF-5) was selected for further studies. On potato dextrose agar (PDA), the isolates produced fast-growing whitish to grey mycelial colonies that became dark with age represent in Fig. 2. Microscopic examination revealed characteristic hyaline to dark brown septate hyphae and typical produced black globose long necked perithecium, fimbriata like ostiole liberating the ascospores, hat shaped ascospores produced, hyalin cylindrical endoconidia, thick walled brown aleurioconidia, confirming the identity of the pathogen as *C. fimbriata* representing in Fig. 3. These

observations are in agreement with earlier reports (Harrington *et al*., 2005; Sharma *et al*., 2010; Chaudhari *et al*., 2016). The pathogen was frequently recovered from vascular tissues exhibiting dark greyish-brown discoloration, indicating its systemic nature and close association with wilt symptoms.

Pathogenicity of *Ceratocystis fimbriata* causing wilt of pomegranate

Artificial inoculation of six-month-old pomegranate seedlings (cv. Bhagva) with *Ceratocystis fimbriata* successfully induced wilt symptoms under greenhouse conditions, thereby confirming the pathogenic nature of the isolate. Typical disease symptoms, including leaf yellowing, drooping and progressive wilting, were observed within 29 days after inoculation. In contrast, control plants treated with sterile distilled water remained healthy and symptomless throughout the observation period. Re-isolation of the pathogen from symptomatic tissues yielded cultures morphologically identical to the original isolate, thus fulfilling Koch's postulates and establishing *C. fimbriata* as the causal agent of pomegranate wilt. The pathogenicity response observed in the present study is consistent with earlier reports describing similar symptom development following artificial inoculation of pomegranate and other woody hosts with *C. fimbriata* (Harrington *et al*., 2005; Sharma *et al*., 2010). These results further corroborate the aggressive and vascular-invading nature of the pathogen, which disrupts water translocation leading to rapid wilt. The confirmation of pathogenicity provided a reliable basis for subsequent studies on pathogen variability and evaluation of biological control agents for the management of pomegranate wilt.

***In vitro* efficacy of *Trichoderma* spp. against *Ceratocystis fimbriata* causing wilt of pomegranate by dual culture technique**

The antagonistic potential of six *Trichoderma* species against *Ceratocystis fimbriata*, the causal agent of pomegranate wilt, was evaluated under in vitro conditions using the dual culture technique. The results revealed significant variation among the tested *Trichoderma* species in suppressing the mycelial growth of the pathogen (Table 2, Fig. 4). The untreated control recorded the maximum radial mycelial growth of 65.00 mm, indicating uninhibited growth of the pathogen. Statistical analysis showed that all treatments differed significantly at the 1% level (CD = 1.81; SE(m±) = 0.43), confirming the effectiveness of Trichoderma spp. as antagonists.

Among the evaluated bioagents, *Trichoderma hamatum* (T_5) exhibited the strongest antagonistic activity, recording the minimum average radial mycelial growth of *C. fimbriata* (15.20 mm) and the highest percent inhibition (76.62%). This was followed by *T. asperellum* (72.36%) and *T. asperelloides* (70.41%), which also showed strong suppression of pathogen growth. Moderate inhibition was observed with *T. koningii* (67.44%) and *T. longi* (61.33%), whereas *T. harzianum* showed the lowest inhibition (58.51%) among the tested species, though it remained significantly superior to the control represented data in Fig 4 and graphically represented in Fig. 5.

The superior antagonistic performance of *T. hamatum* may be attributed to its rapid colonization ability, effective competition for nutrients and space and production of antifungal metabolites and cell wall–degrading enzymes such as chitinases and β-1,3-glucanases. These mechanisms are well documented as primary modes of action of *Trichoderma* spp. against soil- and vascular-borne pathogens (Dennis and Webster, 1971; Howell, 2003; Harman *et al*., 2004). In addition, *Trichoderma* species are known to exhibit mycoparasitism, wherein they coil around and penetrate the hyphae of the pathogen, leading to hyphal lysis and growth suppression (Vinale *et al*., 2008).

The observed variation in antagonistic efficacy among the *Trichoderma* species highlights the importance of species- and strain-specific screening. Such variability has been widely reported and is influenced by genetic makeup, metabolic potential, and adaptation to local agro-climatic conditions (Mukherjee *et al*., 2012). Similar findings on the effectiveness of *T. hamatum, T. asperellum,* and *T. asperelloides* against soil-borne pathogens have been reported earlier by several workers (Howell, 2003; Harman *et al*., 2004; Sharma *et al*., 2013).

Overall, the results of the present study clearly demonstrate that *T. hamatum* is the most promising antagonist against *C. fimbriata*, followed by *T. asperellum* and *T. asperelloides*. These native *Trichoderma* species possess considerable potential for inclusion in integrated disease management strategies for pomegranate wilt. However, further evaluation under field conditions is essential to validate their efficacy and consistency before large-scale application.

Table 2 : *In vitro* efficacy of different *Trichoderma spp.* against *Ceratocystis fimbriata (PCF-5).*

Tr No.	Treatment	Average radial mycelial growth (mm)	Percent Inhibition (%)
T_1	*Trichoderma harzianum*	26.97	58.51
T_2	*Trichoderma longi*	25.13	61.33
T_3	*Trichoderma koningi*	21.17	67.44
T_4	*Trichoderma asperolloid*	19.23	70.41
T_5	*Trichoderma hamatum*	15.20	76.62
T_6	*Trichoderma asperellum*	17.97	72.36
T_7	Control	65.00	0.00
	F Test SE (m±) CD@(0.01%)	Sig. 0.43 1.81	-

Conclusion

The present study confirmed *Ceratocystis fimbriata* as the causal agent of pomegranate wilt in Maharashtra through successful isolation, pathogenicity tests, and symptom reproduction, thereby fulfilling Koch's postulates. In vitro evaluation of native *Trichoderma* spp. demonstrated significant antagonistic activity against the pathogen, with *Trichoderma hamatum* showing the highest mycelial growth inhibition, followed by *T. asperellum* and *T. asperelloides*. The findings clearly indicate that region-specific *Trichoderma* isolates, particularly *T. hamatum*, have strong potential as eco-friendly biocontrol agents and can be effectively integrated into sustainable management strategies for pomegranate wilt, subject to further field validation.

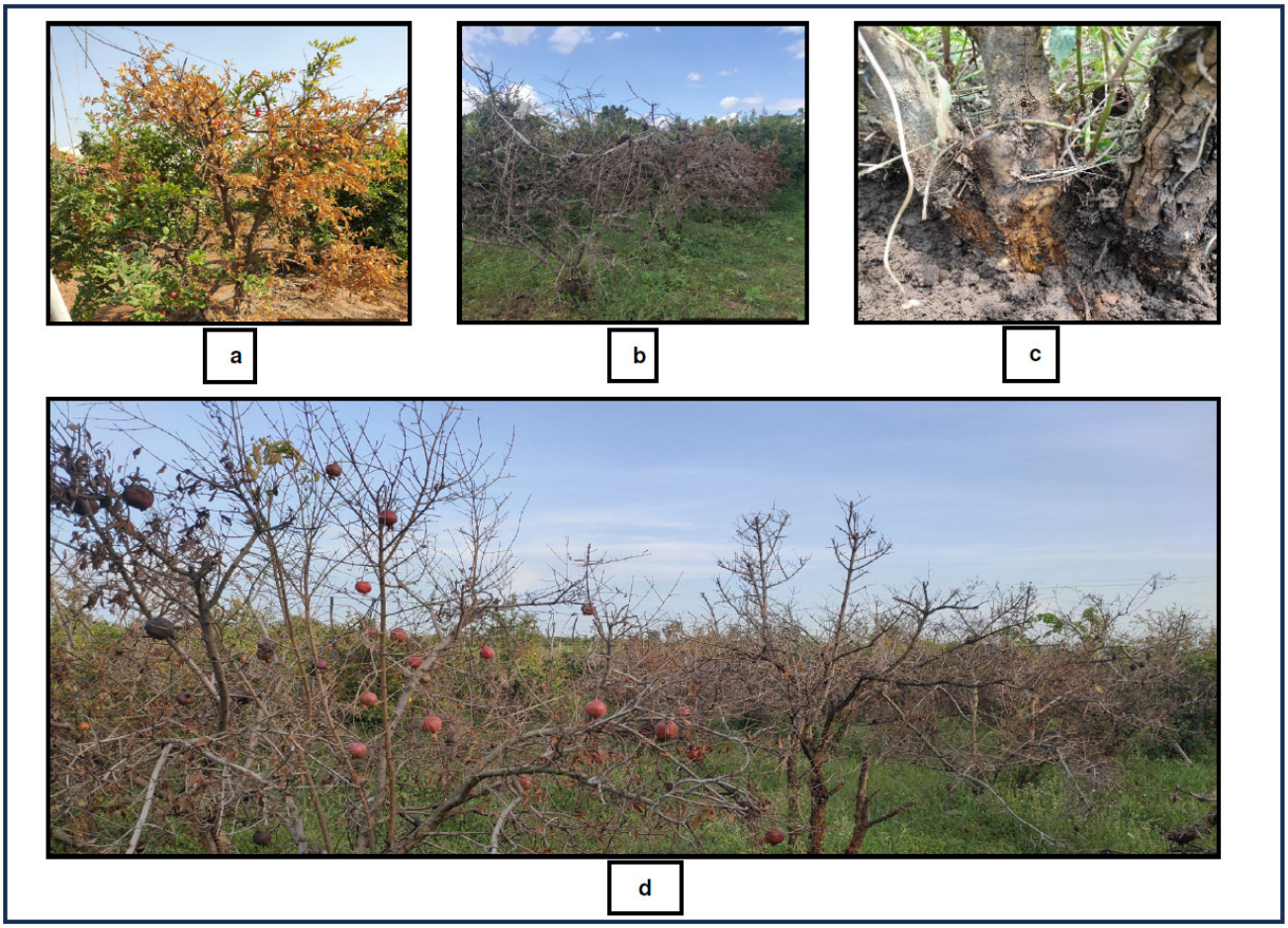

Fig. 1 : Symptomatology of *Ceratocystis fimbriata* causing wilt of pomegranate a) Initially yellowing and partial wilting, b) Complete wilting continuous interval, c) Vertical cracking on stem, d) Complete wilting at fruiting stage

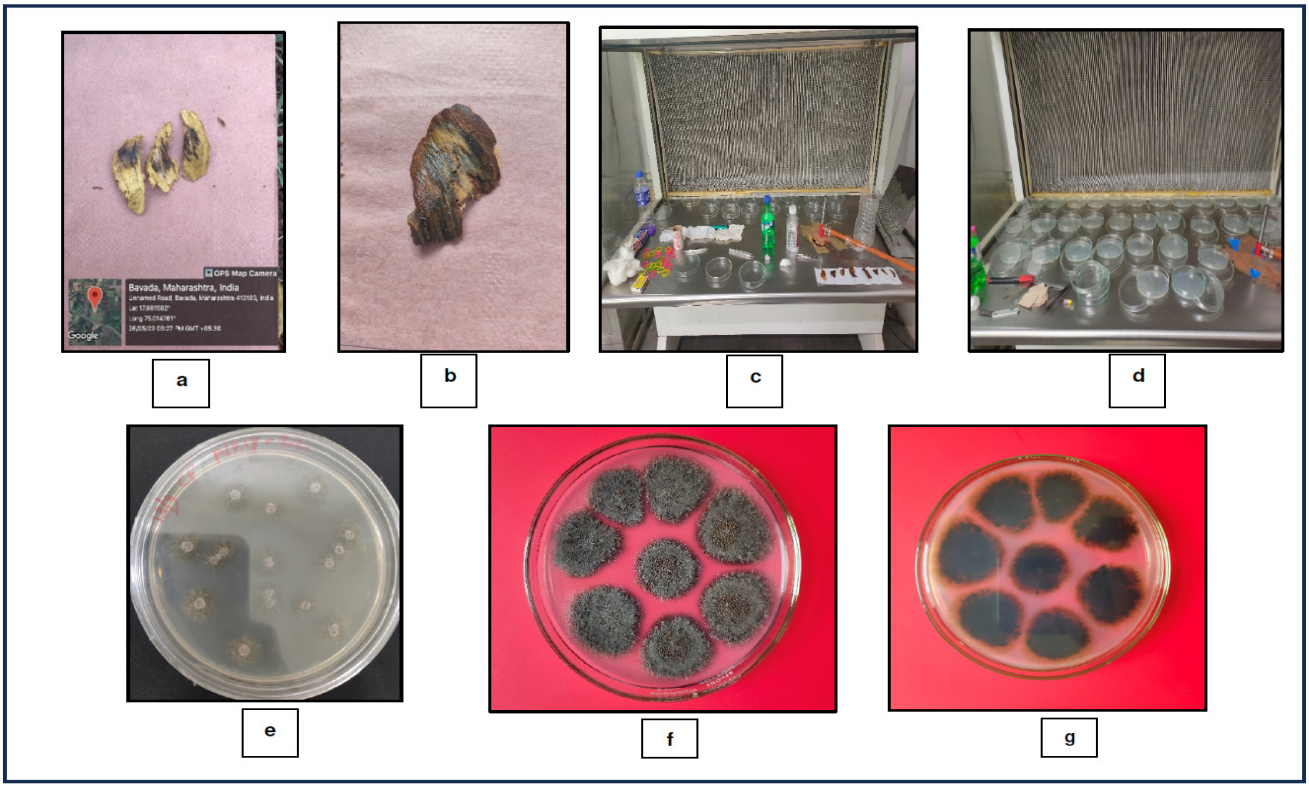

Fig. 2 : Tissue isolation of *Ceratocystis fimbriata* from stem and root tissues of pomegranate. (a-b) Disease infected tissues sample, (c) Diseased sample cut and washed in sodium hypo chloride, (d) PDA media pour in petriplate for isolation. (e-g) Pure growth of *Ceratocystis fimbriata*

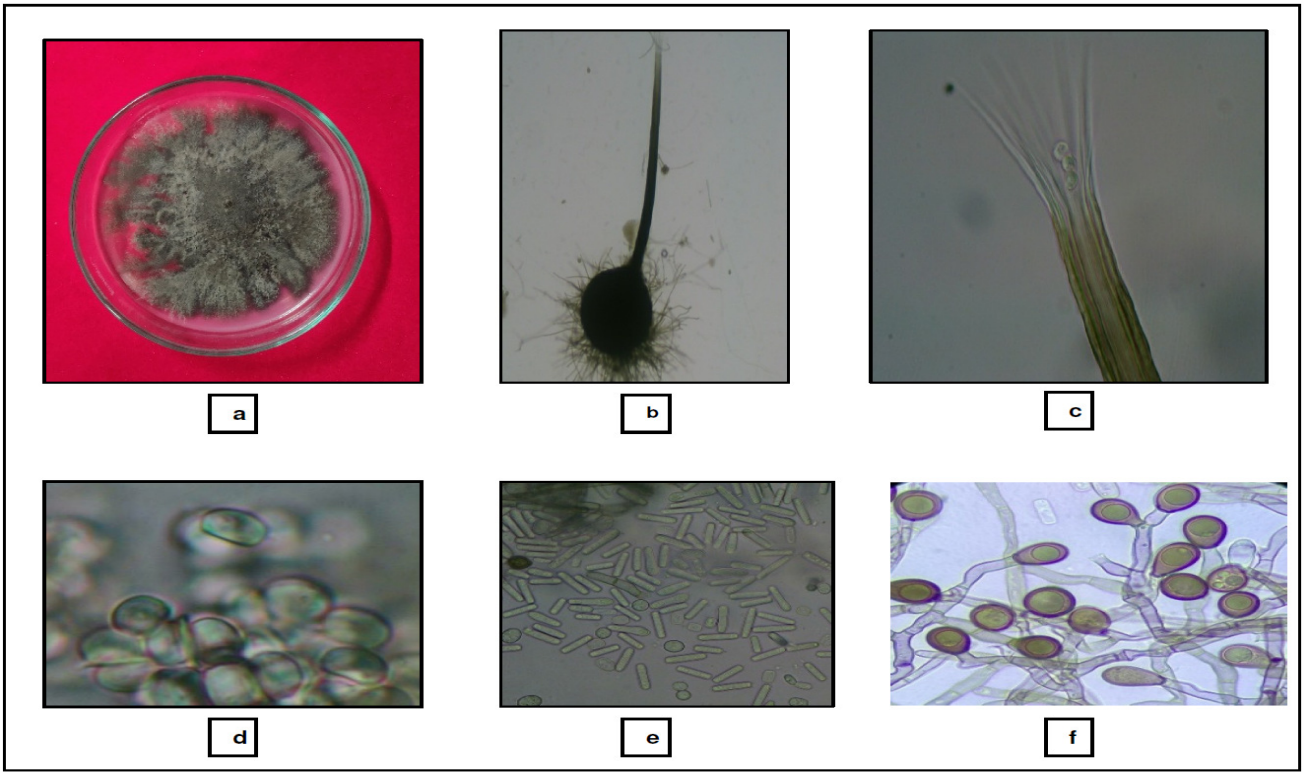

Fig 3. General morphology of *Ceratocystis fimbriata* isolates associated with pomegranate wilt. a) colony morphology, b) Long necked perithecia, c) Ostiole hyphae, d) Hat shaped ascospores, e) Cylindrical endoconidia, f) Thick walled aleurioconidia

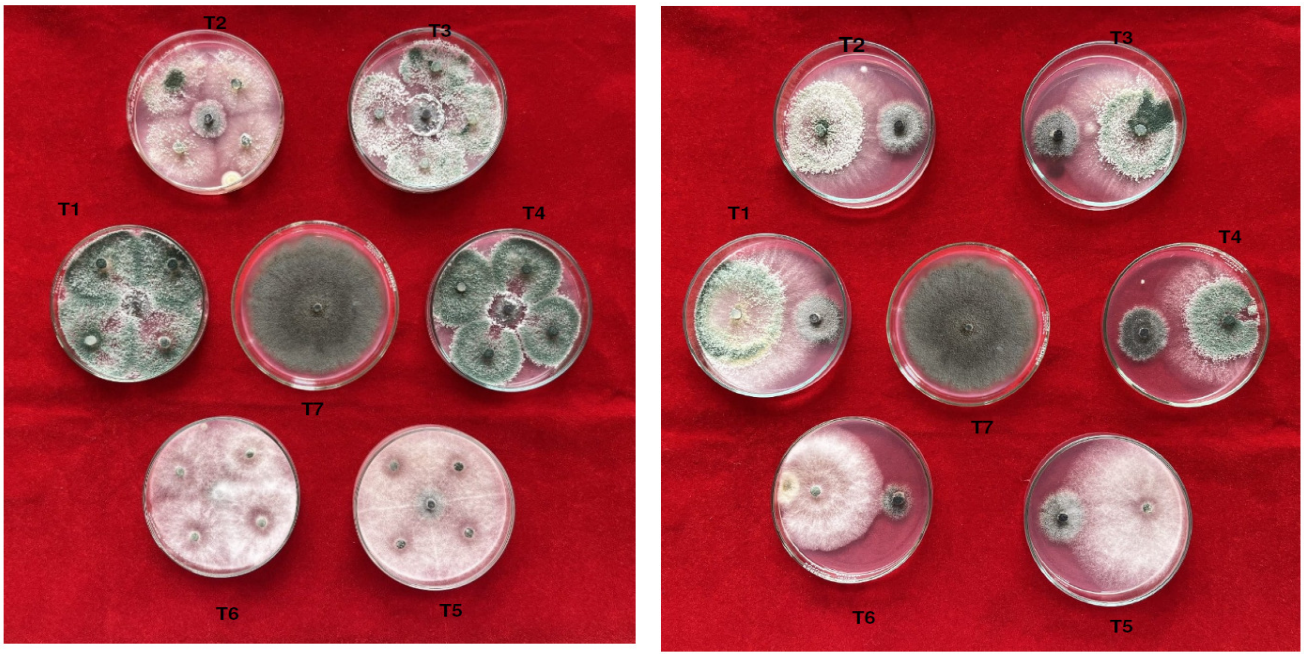

Fig. 4 : *In vitro* efficacy of different *Trichoderma* spp. against *Ceratocystis fimbriata (PCF-5).*

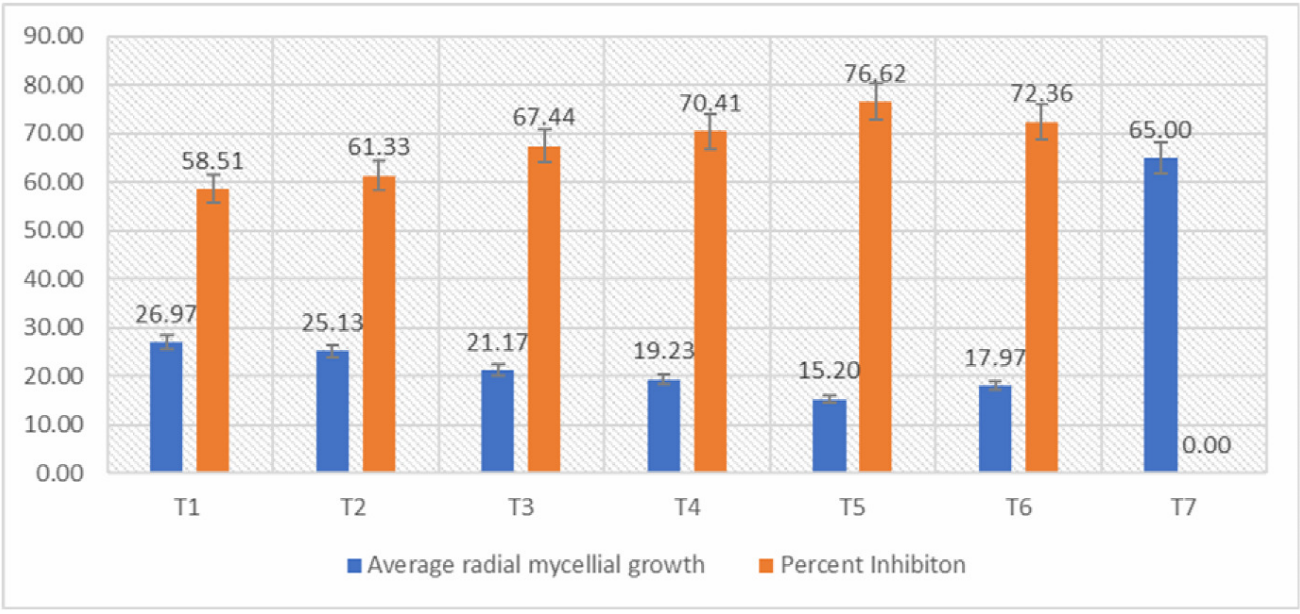

Fig. 5 : *In-vitro* antagonistic activity of *Trichoderma* spp. against *Ceratocystis fimbriata* (PCF-5)

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