Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(5): 1581-1585
Received: 04-07-2019
Accepted: 06-08-2019

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Isolation and bio-efficacy screening of native *Trichoderma* species as a potential biocontrol agents against pomegranate wilt caused by *Ceratocystis fimbriata* Ellis and Halst

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Abstract
The main emphasis of the present study was isolation and antagonistic screening of native *Trichoderma* isolates against *Ceratocystis fimbriata* Ellis and Halst. The causal agent of wilt of pomegranate, which cause estimated losses up to 30%. Thirty three native *Trichoderma* spp. were isolated from the rhizosphere soil of healthy pomegranate plants, identified using morphological and microscopic characteristics and were evaluated for *in vitro* antifungal activity against *Ceratocystis fimbriata* by dual culture plate technique. Among the Thirty three isolates maximum per cent inhibition of 93.10 per cent was observed in *Trichoderma harzianum* (PT-11 isolate) followed by *Trichoderma viride* (PT-10) and *Trichoderma harzianum* (PT-4) of about 92.03% and 91.66%, respectively when compared to the all other isolates of *Trichoderma* spp. Based on the dual culture technique, eleven superior native *Trichoderma* isolates were selected for elucidating volatile metabolites production by paired plate method. The inhibition of pathogen mycelium varied depending on the *Trichoderma* species producing volatile metabolites, *Trichoderma virense* (PT-6) inhibited fungal growth up to 78.84% followed by *Trichoderma harzianum* (PT -11) of about78.67% and *Trichoderma viride* (PT-10) of 71.84%. Among superior eleven isolates, PT-6, PT-10 and PT-11 isolates were used for growth promotion and disease control studies in pot culture experiment under greenhouse. The growth of pomegranate seedlings with the antagonist alone and in combination with pathogen was greater than in plants were inoculated with pathogen alone. Among all isolates, *T. virense* (PT-6) showed higher mean shoot and root length (45.70 cm and 19.04 cm) with lowest disease incidence (20.38%) when compared to highest incidence in pathogen check (41.20%).

Keywords: Pomegranate, wilt, *Ceratocystis fimbriata*, *Trichoderma* spp.

Introduction
Pomegranate (*Punica granatum* L.) is a vital cash crop of India, is commercially cultivated in the states of Maharashtra, Karnataka, Andhra Pradesh, Gujarat, Rajasthan and Tamil Nadu. In recent years, wilt disease caused by *Ceratocystis fimbriata* Elli. and Halst is becoming a major threat by adversely affecting the yields of the pomegranate fruit crop (Sharma *et al*., 2008 and Sharma, 2009) [23, 24]. The disease was first reported from Nashik district in Maharashtra in 1978 and subsequently from Kaladgi and Kanamadi areas of Karnataka in 1988 (Somasekhara, 1999) [25] and Cudddapa, Andhra Pradesh in 2002. The disease has also been reported from other countries like Iran (Banihashemi, 1998) [1], China (Huang *et al*., 2003) [14] and Greece (Tziros and zavella-Klonari, 2008) [32]. Based on the damage and loss, the disease has been considered as a nationally important disease in India and lot of emphasis has been given to tackle this disease problem. However, there is no much research work carried out with regards to biological control of wilt of pomegranate. *Trichoderma* sp. is one of the most important biocontrol agent used for management of different diseases (Harman, 2004) [10]. *Trichoderma* spp. are free living fungi that are common in soil and root ecosystems and promote plant growth (Yedidia, 2001) [36]. *Trichoderma* spp. are effective in control of soil borne fungal diseases in several crop plants (Kubicek *et al*., 2001) [17]. Different isolates of *Trichoderma* spp. were identified as biocontrol agents of groundnut stem rot and other soil-borne diseases (Podile and Kishore, 2002) [19]. These free-living fungi are ubiquitous in the soil environment and are being successfully used and commercialized to combat a broad range of phytopathogenic fungi (Hjeljord *et al*., 2000 [12]; Desai *et al*., 2002 [5]; Fravel, 2005 [9]). *Trichoderma* spp. can directly impact other fungi, after sensing a suitable fungal host, *Trichoderma* sp. responds with the production of antibiotic compounds, formation of

specialized structures and degradation of the host's cell wall, followed by the assimilation of its cellular content, a process known as mycoparasitism (Steyaert *et al*., 2003 [29]; Benitez *et al*., 2004 [2]). The mechanisms of mycoparasitism, antibiosis and competition afforded by *Trichoderma* spp. have been widely studied (Howell, 2003 [13]; Harman *et al*., 2004) [10]. Application of bio-agents for growth promotion and disease management has attracted much attention in the past few decades, due to serious environmental and human health problems resulting from the application of chemical pesticides (Cook, 1993 [3]; He, 1993) [11]. Chemical fungicides offer a degree of protection against pathogens, but their adverse effect on beneficial soil microorganisms and the environment cannot be ignored. In this context, biocontrol agents appear to hold promise in plant growth promotion and disease management. Using *Trichoderma* spp. control of soil borne plant pathogens has been reported by many investigators (Ulkhede, 1992 [33], Ziedan *et al*., 2005 [37]) but there is little information was available on the use of *Trichoderma* spp. as biocontrol agents against *C. fimbriata* in pomegranate plants. The objective of the present investigation was isolation and screening of effective native isolates *Trichoderma* spp. against the wilt pathogen *C. fimbriata* and elucidation of volatile metabolite production mechanism of *Trichoderma* spp.

Materials and Methods

Source and isolation of *Trichoderma* spp.

Trichoderma spp. was isolated from rhizosphere soil by serial dilution technique (Krassilnikov, 1950) [16] on *Trichoderma* specific medium (Elad and Chet, 1981) [7]. Ten grams of healthy rhizosphere soil samples was taken separately and suspended in 90ml of sterile water and stirred well to get 1: 10 dilution (10^{-1}). One ml from this was transferred to test tubes containing 9ml of sterile distilled water to get 1:100 (10^{-2}) dilution. Likewise the dilution was made up to 10: 10000 (10^{-4}). One ml of the final dilution of each sample was aseptically transferred into petri plates containing *Trichoderma* specific medium, the plates were incubated at 25± 1˚C for 5- 10 days. Fungus was sub cultured on potato dextrose agar slants and allowed grow at 25 ºC for 15 days and such slants were preserved in a refrigerator at 4 ºC and sub cultured once in 30 days.

Three healthy rhizosphere soil samples were collected from each taluk and totally thirty three representative *Trichoderma* spp. were isolated from five districts surveyed.

Screening for antagonistic activity of native *Trichoderma* spp. isolates

All the thirty five isolates were screened for potential antagonistic activity against pathogenic fungus *C. fimbriata* using dual culture technique. All antagonistic pathogen combinations were examined on 20ml of PDA in 9 cm petriplates, with three replicate plates per treatment. For dual culture technique, a mycelial disc (5mm in diameter), taken from actively growing 8 day old culture of *C. fimbriata* and 3 days old *Trichoderma* isolates placed 8cm apart from each other on the PDA. For control treatments, a 5mm disc of *C. fimbriata* was placed on the PDA medium. The plates were incubated at 28ºC for 8 days. Observations on the antagonistic activities of *Trichoderma* isolates on *C. fimbriata* were determined by measuring the radial growth of pathogen with fungal antagonistic culture and control. The per cent inhibition over the control was calculated by using the formula (Vincent, 1947).

Per cent inhibition (I) = C-T/C ×100

Where, C- mycelial growth of pathogen in control

T- Mycelial growth of pathogen in dual plate

Effect of volatile compounds produced by antagonists on the radial growth of *C. fimbriata*

Volatile compound production assay was done by following the method of Dennis and Webster (1971) [4]. Eleven different species of *Trichoderma* were inoculated in the center of the petri plates containing solidified sterilized PDA medium by placing 6mm disc (3d old culture) from the margin of the actively growing region of *Trichoderma* spp. and incubated for 3 days at 28±1 ºC. After that the top lid of each petri plate was replaced with bottom part of another petri plate with same size containing PDA medium, duly inoculated with a 6mm mycelial disc of the test pathogen after 3 days of incubation. The pairs of each plate were sealed with parafilm (adhesive tape) and incubated at 28±1 ºC. The PDA medium without *Trichoderma* isolate in the bottom part of petri plate with respective test pathogen on the upper lid of plate served as control. Three replicates were maintained for each treatment. The assembly was opened after 72 h. and the observations were recorded by measuring colony diameter of the test pathogen (in mm) in each plate and that of the control plates.

Pot culture technique

Potato dextrose broth was prepared in 250ml conical flasks. Each flask inoculated with different efficient isolates of *Trichoderma* spp., separately and incubated at 28± 2 ºC for 10 days. 1kg of soil and 1kg of sand was taken into polythene bags and sterilized at 121 ºC for 30 min at 15 lbs pressure for two successive days. 9" earthenware pots were taken; sterilized sandy soil was added into the pots. The talc based formulation of *Trichoderma* spp. was prepared according to the method described by Jayarajan and Ramakrishnan (1991) [15]. Nine mm disc of *Trichoderma* spp. was inoculated into 100 ml potato dextrose broth and incubated at room temperature (28± 2 °C) for 5 days. The mycelial mat was mixed with talc powder in 1:2 ratio and shade dried. To this, carboxy methyl cellulose was added at the rate of 0.5 percent as sticker. The product was shade dried to 20 per cent and packed in polypropylene bags and sealed.

Three months old pomegranate seedlings were planted in pots filled with sandy soil containing *C. fimbriata*. Different talc based formulation of superior isolates of *Trichoderma* spp. was applied after 30 days after planting and three replicates were maintained for each treatment. Control pots also maintained without any fungal cultures. For recording wilt incidence, observations on total number of branches and number of wilted branches in each treatment were recorded. Later, per cent disease incidence was calculated.

Results

Biological control of plant diseases using microbial inoculants is receiving increased attention as an eco-friendly alternative to chemical pesticides. Exploitation of plant-microbe interaction in the rhizosphere could promote plant growth and reduces the diseases.

Isolation and Identification of *Trichoderma* isolates

Native *Trichoderma* spp. were isolated by serial dilution technique on *Trichoderma* specific medium (TSM) and incubated for 7 days at 25 ± 2 ºC. After incubation, all the thirty three isolates showed typical character of greenish

fungal colony on TSM and produced fungal characters of *Trichoderma* under microscope. The *Trichoderma* isolates were obtained and pure culture of isolates was maintained on PDA slants for further studies.

Effect of native isolates of *Trichoderma* species on mycelial growth of *C. fimbriata in vitro*

A total of Thirty three isolates of *Trichoderma* were obtained from healthy rhizosphere soil of wilt affected orchards. Among these thirty three isolates only eleven were bioactive and have been reported in the present study. The isolates which showed more than 50% of mycelial inhibition were selected as efficient superior isolates and designated as PT-1, PT-2… and PT -11.

Thirty three isolates of *Trichoderma* spp. were screened against *C. fimbriata* for mycelial inhibition by dual culture technique. Zone of inhibition of mycelium (in mm) was recorded and the per cent inhibition was calculated.

The results revealed that per cent inhibition of mycelial growth varied greatly among the thirty three isolates. However, the per cent inhibition was higher (> 50%) in eleven isolates which varied from 80.70 to 93.10 per cent. The results obtained were highly significant between the different isolates (PT-1….PT-11) and also over control. Maximum per cent inhibition of 93.10 per cent was observed in PT-11 followed by PT- 10 (92.03%) and PT-4 (91.66%). Least inhibition of 78.63 per cent was observed in PT-8 followed by PT-2 (80.70%).

Table 1: Antagonist activity of native *Trichoderma* isolates against *C. fimbriata*

Sl. No.	Isolate	Radial mycelial growth(mm)	Per cent mycelial Inhibition*
1	PT-1	16.00	82.20 (65.04)
2	PT-2	17.16	80.70 (63.93)
3	PT-3	13.46	85.10 (67.29)
4	PT-4	7.46	91.66 (73.21)
5	PT-5	9.01	90.00 (71.56)
6	PT-6	10.0	88.83 (70.47)
7	PT-7	7.16	92.00 (73.57)
8	PT-8	19.16	78.63 (62.46)
9	PT-9	12.83	85.62 (67.71)
10	PT-10	7.16	92.03 (73.60)
11	PT-11	6.16	93.10 (74.77)
12	Control	90.00	0.00
S.Em±			0.27
C.D. at 1%			1.06

***** Mean of three replications
Figure in parenthesis are arcsine value

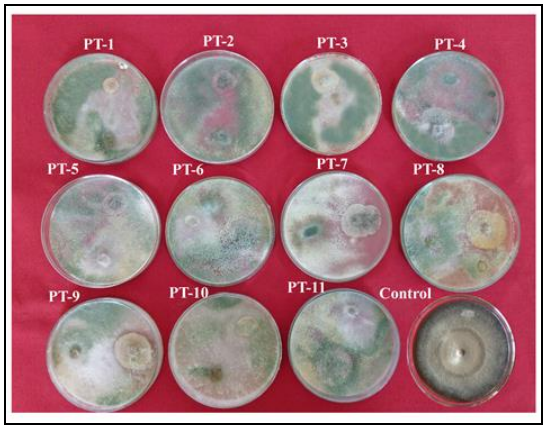

Plate 1: Antagonistic activity of native *Trichoderma* spp. isolates against *C. fimbriata*

Volatile compounds produced by *Trichoderma* spp. against *C. fimbriata*

Eleven superior native *Trichoderma* isolates were tested for volatile compound production assay in paired plate technique. The results revealed that the isolates produced considerable amount of volatile metabolites which varied with the isolates. Higher concentrations of volatile metabolites were produced in isolate PT-6 (78.84%) followed byPT-11 (78.67%), FP-10 (71.84%) and lowest concentration of volatile metabolites was produced by PT-8 (50.00%). All the isolates have shown significant difference in mycelial growth inhibition when compared to the uninoculated control.

Table 2. Production of volatile metabolites from efficient native Trichoderma isolates on mycelial inhibition of C. fimbriata

Sl. No.	Isolate	Identification	Radial mycelial growth (mm)	Per cent mycelial inhibition*
1	PT-1	*Trichoderma viride*	29.60	63.10(52.60)
2	PT-2	*Trichoderma virens*	31.20	58.80(50.06)
3	PT-3	*Trichoderma viride*	29.30	60.70(51.17)
4	PT-4	*Trichoderma harzianum*	20.00	70.00(56.78)
5	PT-5	*Trichoderma hamatum*	21.60	68.40(55.79)
6	PT-6	*Trichoderma virens*	11.16	78.84(62.61)
7	PT-7	*Trichoderma harzianum*	23.76	66.24(54.47)
8	PT-8	*Trichoderma virens*	40.00	50.00(45.00)
9	PT-9	*Trichoderma harzianum*	34.13	55.87(48.37)
10	PT-10	*Trichoderma viride*	18.16	71.84(57.94)
11	PT-11	*Trichoderma harzianum*	11.33	78.67(62.49)
12.	Control	-	90	0.00
	S.Em±			0.42
	C. D. @ 1%			1.26

* Mean of three replications
Figure in parenthesis are arcsine value

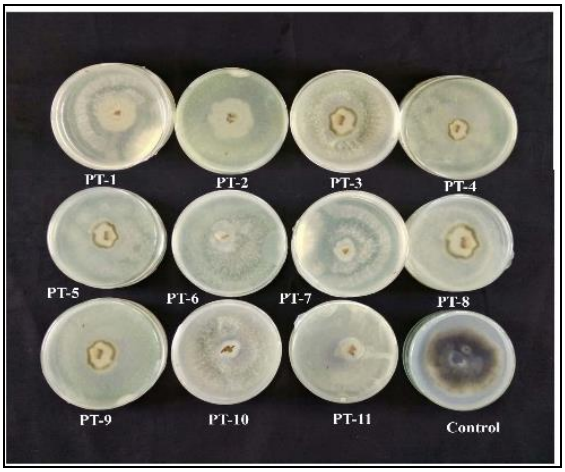

Plate 2. Volatile metabolites production by efficient *Trichoderma* spp. isolates

Discussion

In this preliminary investigation, an attempt was made to screen the antagonistic potential of rhizospheric native bioagents against *C. fimbriata*, the causal organism of wilt disease in pomegranate. Earlier reports attribute *Trichoderma* spp. as one of the most efficient strains to be evaluated as biocontrol agent.

All the thirty three isolates of *Trichoderma* spp. were showed antagonism against *C. fimbriata*. However, only eleven isolates showed high level of antagonistic activity by recording more than 50 per cent inhibition of test pathogen. Among the eleven superior isolates, the per cent inhibition of mycelium varied from 80.70 to 93.10 per cent. Maximum per cent inhibition of PT-11 (93.10%), followed by PT- 10 (92.03%) and PT-4 (91.66%). Least inhibition of 78.63 per cent were observed in PT-8 followed by PFP-2 (80.70%). Similar trend was observed earlier with *T. harzianum* against *C. paradoxa* (Vijaya *et al*., 2007) [24]. Sonyal *et al*. (2015) [26] who reported *T. harzianum* and *T. viride* showed maximum inhibition of the test fungus (100%) and *T. harzianum* (Th-R) and Diamond (*T. viride*) recorded maximum inhibition of (100%) mycelial growth of *C. fimbriata* (Raja, 2017) [20]. Several authors have also reported that *Trichoderma* spp. inhibited several plant pathogens including *C. fimbriata* (Sampang, 1989; Eziashi *et al*., 2006; Talukder *et al.*, 2007 and Padmaja *et al*., 2013) [22, 8, 31, 18].

Eleven native *Trichoderma* isolates were showed various concentration of volatile compound production in paired plate technique. Higher concentrations of volatile metabolites were produced in isolate PT-6 (78.84%) followed by PT-11 (78.67%), FP-10 (71.84%) and lowest concentration of volatile metabolites was produced by PT-8 (50.00%).

The findings were in accordance with Sreedevi *et al*. (2013) [27] they evaluated effective *Trichoderma* spp. for biocontrol of *Macrophomina phaseolina* the causative agent of root rots of groundnut. The 24 h old culture of *T. harzianum* produced maximum volatile metabolites which accounted for 64.70 per cent inhibition of growth of*M. phaseolina* followed by *T. viride* (47%). Ten isolates of *Trichoderma* spp. evaluated for the production of volatile compounds. Among the various isolates *T. viridae* showed maximum mycelial inhibition against *Sclerotium rolfsii* (69.33%) (Rekha, 2010) [21]. Similar results also reported earlier by Srivatsava and Singh, 2000; Divya, 2015) [28] [6].

The application of *Trichoderma* native antagonists through soil application was found effective in suppressing wilt incidence (20.38%). Also the results of this experiment revealed that the application of native *Trichoderma* spp. increased shoot and root length of seedlings. Among all *Trichoderma* isolates PT-6 showed higher shoot and highest root growth (45.70 cm and 19.04 cm respectively). Similar results were recorded by Sundaramoorthy and Balabaskar (2013) [30] application of native *Trichoderma* antagonists through seedling dip and soil application was found effective in suppressing wilt incidence (15.33 to 25.50%). Conspicuously, an application of ANR-1 antagonistic fungal formulation was recorded least wilt incidence (15.33%) followed by KGI-3 (17.45%) compared to other isolates.

Conclusion

Trichoderma spp. are effective biocontrol agents for a number of soil borne plant pathogens and plays a major role in controlling plant diseases (Yedidia *et al*., 1999) [36]. *Trichoderma* species are among the most-promising biocontrol fungi against many fungal plant pathogens. In our present study, we have isolated and screened native isolates of *Trichoderma* spp. against wilt disease of pomegranate caused by *C. fimbriata*. *Trichoderma* isolates are more effective and show excellent control of *C. fimbriata*, responsible for Pomegranate wilt. These isolates could be exploited for their volatile compound production mechanism. The three superior isolates will be promising bio control agent against wilt and plant growth promotion activity in pomegranate seedlings. But there is still need to work in future regarding biotechnological approaches for effective and eco-feasible control of wilt disease.

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