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Bio efficacy of bioagents against *Ceratocystis fimbriata* ELL. & Halst and *Fusarium oxysporum* Schlecht causing wilt of pomegranate

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Abstract
Wilt disease of Pomegranate caused by *Ceratocystis fimbriata* and *Fusarium oxysporum is* becoming a serious threat in recent years. In order to find the preventive measures, efficacy of seven different fungal bioagents and five bacterial antagonists were evaluated under *in vitro* condition. Among all bioagents, *Trichoderma viride* showed maximum mycelial inhibition (87.55%) of *C. fimbriata* followed by *T. harzianum* isolate 69 (83.53%). Whereas against *F. oxysporum*, maximum inhibition was observed in *T. koningiopsis* (71.43%) followed by *T. viride* (67.86%) and *T. asperillium* isolate 07 (67.86%). Among bacterial antagonists, *Bacillus amyloliquifaciens* gave maximum inhibition of 76.31% and 60.71% against *C. fimbriata* and *F. oxysporum*, respectively.

Keywords: Pomegranate, wilt, bioagent, *Ceratocystis*, *Fusarium*

Introduction
Pomegranate (*Punica granatum* L.) commonly known as *Dadam* or *Anar* belongs to the family Punicaceae, is regarded as the "Fruit of Paradise". The scientific name *Punica granatum* is derived from the name *Pomum* (apple) *granatus* (grainy), or seeded apple. It is originated in Iran and became popular in Iraq. It is one of the first five domesticated edible fruit crops along with fig, date palm, grape and olive. It is widely cultivated in Mediterranean, tropical and subtropical regions and is considered as an excellent fruit crop for growing in arid zones for its tolerance to drought conditions. It is mostly cultivated in Spain, Morocco and other countries around the Mediterranean, Egypt, Iran, Afghanistan, Arabia, Baluchistan and some extent in Burma, China, Japan, U.S.A. (California) and India.

At the global level, India is the world's largest producer of pomegranate with 743.1 thousand tonnes followed by Iran with 650 thousand tonnes. India is the only country in the world where pomegranate is available throughout the year. In India, it is regarded as a "vital cash crop", grown in an area of 2, 08, 730 ha with a production of 24, 42, 390 tonnes with an average productivity of 11.70 MT (Anon., 2017) [1]. Among the different states Maharashtra is the largest producer of pomegranate occupying 2/3rd of total area in the country followed by Karnataka, Andhra Pradesh, Gujarat and Rajasthan (Jayesh and Kumar, 2004) [7]. Karnataka state has the distribution of cultivating pomegranate under tropical condition in an area of 28, 090 ha with a production of 3, 28, 920 tonnes with an average productivity of 11.71 MT (Anon., 2017) [1]. In Karnataka this crop has spread across different districts *viz*., Bijapur, Bellary, Bagalkot, Koppal, Chitradurga, Belgaum, Davangere, Gadag, Tumkur, Bangalore and Gulbarga.

Nowadays pomegranate cropping is largely limited by biotic stresses. Among the diseases infecting pomegranate, the wilt complex disease popularly known as 'die back and sooragu rooga' caused by either *Ceratocystis fimbriata/ Fusarium oxysporum/Meloidogyne incognita* or as a complex is the major production constraint. Pomegranate wilt complex is one of the important diseases of pomegranate adversely affecting crop cultivation in all major growing regions of India. It was first reported from Nasik district of Maharastra in 1978 and was subsequently noticed in two areas of the Bijapur district of India in 1990. Around 1993, rapid spread of this disease was observed in the entire Bijapur district. The cause was not identified until 1995, in 1996 the fungus *C. fimbriata* was isolated from discolored stem, root, and branch tissues on wilted plants. The disease is prevalent in parts of a Maharashtra, Karnataka, Andhra Pradesh,

Gujarat and Tamil Nadu states (Somasekhara *et al.*, 2000 [13], 2009 [15]; Jadhav and Sharma, 2009 [6]). Somasekhara (1999) [14] reported wilt of pomegranate was caused by *C. fimbriata* in India. Chavan and Dake (2001) [2] collected the roots of wilt infected pomegranate plants from Ahemdnagar and Solapur districts of Maharashtra, and identified the fungus as *F. oxysporum*.

Chemical control though necessary for wilt disease at present, are undesirable and even inadequate as a long-term solution to crop and soil health. So it is important to generate information on the efficacy of available bioagents for managing the disease as one of disease management practice. Hence, the present study was undertaken to screen biogents under *in vitro* condition to manage wilt.

Materials and methods

Isolation of Pathogen From Infected Plant Parts

Ceratocystis fimbriata, associated with wilt was isolated from the infected stems of pomegranate plant. Stem portions with characteristic symptoms of vascular staining were collected and sliced piece was surface sterilized with 1% sodium hypochlorite for about 2 minutes and twice with sterile water to remove traces of sodium hypochlorite. Pathogen isolation was made using carrot bait technique in which, stems were placed in between the carrot disks and kept in a humid chamber and incubated at 25 ± 2 °C under 12 hour photoperiod (Moller & DeVay, 1968) [9]. After perithecium formation, a portion of these fungi was transferred to freshly prepared potato dextrose agar to allow the full development of fungi.

The typically infected root and stem portions near the collar region were cut into small pieces with the help of sterilized knife and again washed with the sterilized water. These pieces were then surface sterilized with 1% sodium hypochlorite for about 2 minutes and twice with sterile water. Such pieces were then transferred aseptically onto PDA medium in petri dishes. The petri dishes were incubated for seven days at 27 ± 2°C temperature and were observed periodically for fungal growth. The pathogen obtained from the pieces was transferred aseptically onto PDA slants and incubated for further growth.

Purification of the Pathogen

The culture of pathogen *C. fimbriata* and *F. oxysporum* were purified by standard hyphal tip isolation procedure and then purified culture was maintained on potato dextrose agar slants and kept in a refrigerator at 5°C, for further use in the laboratory and pot culture studies.

Evaluation of Bioagents

Totally twelve different biocontrol agents (Table 1.) were tested against *C. fimbriata* and *F. oxysporum.* Among them some are procured from different institutes and some are isolated from commercial products which is available in market.

In dual culture technique, twenty ml of sterilized and cooled PDA was poured into sterilized Petri plates. Fungal antagonists were evaluated by inoculating the pathogen at one side of Petri plate and the antagonists inoculated at exactly opposite side of the same plate by leaving 3-4 cm gap. In case of bacterial antagonist evaluation, mycelial discs of pathogen were inoculated at one side and bacterial antagonists were streaked at other side of the plate. For this, actively growing cultures were used with three replications. After required period of incubation *i.e.,* after growth of colony in control plate reached periphery, the radial growth of pathogen in treated plate was measured. Per cent inhibition over control was worked out according to formula given by Vincent (1947) [19].

$$I = \frac{C-T}{C} \times 100$$

Table 1: List of antagonists used in the study and with their source

S. No.	Bio-agents	Source
1.	*Trichoderma koningiopsis*	TNAU, Coimbatore
2.	*Trichoderma harzianum*	IOF, Dharwad
3.	*Trichoderma harzianum*	Multiplex Nisarga
4.	*Trichoderma viride*	Kalpavruksha
5.	*Trichoderma asperellum* isolate 07	UAS, GKVK, Dept. of Microbiology
6.	*Trichoderma harzianum* isolate 69	UAS, GKVK, Dept. of Plant pathology
7.	*Trichoderma harzianum* isolate 78	UAS, NBAIR
8.	*Bacillus subtilis*	UAS, Raichur
9.	*Bacillus amyloliquifaciens*	TNAU, Coimbatore
10.	*Bacillus subtilis*	IOF, Dharwad
12.	*Pseudomonas fluorescens*	UAS, Raichur
13.	*Pseudomonas fluorescens*	UAS, Dharwad

Result

Twelve bio control agents (7 fungi and 5 bacteria) were evaluated against *C. fimbriata* and *F. oxysporum.* The per cent growth inhibition of pathogen and antagonistic potential were presented in Table 2- 3 and depicted in plate-1, 2. Efficacy of bioagents against both the pathogens ranged from 36.51 to 87.555 per cent.

The results showed that the antagonists significantly inhibited the growth of *C. fimbriata* and *F. oxysporum* either by overgrowing or by exhibiting inhibition zones.

Maximum reduction in colony growth against *C. fimbriata* was observed in *T. viride* (Multiplex Nisarga) (87.55) which was significantly superior over all other bioagents tested. *Pseudomonas fluorescens* (IOF, Dharwad) showed least inhibition of 51.81 per cent. *T. koningiopsis* (TNAU) showed the maximum mycelial inhibition of 71.43 per cent against *F. oxysporum* which was followed by *T. viride* (Multiplex Nisarga) (67.86%) and *T. asperillium* isolate 07 (UASB) (67.86%). Among five bacterial bioagents *Bacillus amyloliquifaciens* (TNAU) showed maxium inhibition of 76.31 and 60.71 per cent respectively, against *C. fimbriata* and *F. oxysporum. Pseudomonas fluorescens* (IOF, Dharwad) showed least inhibition of mycelial growth against both the pathogens. Considering fungal and bacterial bioagents separately *T. viride* (Multiplex Nisarga) and *Bacillus*

amyloliquifaciens (TNAU) were very effective against both the pathogens.

Discussion

The biological control mechanism operates by way of mycoparasitism (it mainly involves action of antibiosis and cell wall degrading enzymes such as chitinase, β 1-3 glucanases and proteases), antibiosis, competition, induced resistance and inactivation of host enzymes. In the present study, the efficacy of seven fungal and five bacterial bioagents were tested against *C. fimbriata* and *F. oxysporum*.

Among fungal bio agent highest inhibition was observed in *T. viride* and *T. koningiopsis* respectively, against *C. fimbriata* and *F. oxysporum. Trichoderma* having activity of mycoparasitism, antibiosis and competition for resources and space. *Trichoderma* sp. produce a rich mixture of antifungal enzymes, including chitinases and β 1-3 glucanases. These enzymes help to break cell wall of other pathogenic fungi (Harman, 2006) [5]. In general, species of *Trichoderma viz. T. viride, T. koningiopsis, T. harzianum and T. asperellum* showed higher inhibition of pathogens as compared to bacterial antagonists. Sharma (2009) [17] revealed that *T. viride* bio-formulation gave maximum growth inhibition of *C. fimbriata* under *in vitro* condition. Rahman *et al.* (2009) [10] reported that *T. harzianum* IMI-392432 has the most potential to control the *C. paradoxa.* Sonyal *et al*. (2010) reported that among bio-agents *T. harzianum* and *T. viride* showed maximum inhibition of *C. fimbriata* fungus (100%). Imran Khan *et al.* (2017) [5] tested different bio agents against *C. fimbriata*, among them *T. harzianum* showed maximum mycelial inhibition (88.77 %) followed by *T. viride* (86.60 %) and *P. fluorescens* (66.33 %). Raja (2017) [11] reported that *T. harzianum* (Th-R) and *T. viride* (Diamond) showed maximum inhibition of 100 per cent mycelial growth of *C. fimbriata*. Suarez *et al*. (2009) [18] found that *T. koningiopsis* Th003 have ability to control *F. oxysporum* f. sp. *radicis-lycopersici* by inducing systemic defense responses in tomato plants. And the results obtained by using fungal antagonists against *F. oxysporum* are inconfirmation with the work of Kumari *et al*. (2014), Sumana *et al*. (2012) and Redda *et al.* (2018) [12].

Among bacterial bioagents *Bacillus amyloliquifaciens* (TNAU) showed maxium mycelial inhibition against *C. fimbriata* and *F. oxysporum*, and was followed by *Bacillus subtilis* (UASR). Dong-jing *et al* (2018) [3] used *Bacillus amyloliquefaciens* XZ-1 aginst *C. fimbriata* the causal agent of sweet potato black rot. They attributed that XZ-1 strain could induce no obvious swelling or abnormal effects on *C. fimbriata* mycelium, but could change the sporulation type. Zhang *et al*. (2014) [22] evaluated the Strain B37 of *Bacillus subtilis* and found that it was the best biocontrol bacterium on poplar sapstain (*Ceratocystis adiposa* Hz91, *L. theobromae* YM0737, *L. theobromae* Fx46, and *Fusarium* sp., YM05). Yuan *et al.* (2012) revealed that *Bacillus amyloliquefaciens* NJN-6 produces volatile compounds (VOCs) that inhibit the growth and spore germination of *Fusarium oxysporum* f. sp. *cubense*.

As an integral part of integrated management of this disease in pomegranate bioagents along with the judicious use of fungicides can promote the environment friendly management for wilt complex in pomegranate.

Table 2: *In vitro* evaluation of different biocontrol agents against *Ceratocystis fimbriata*

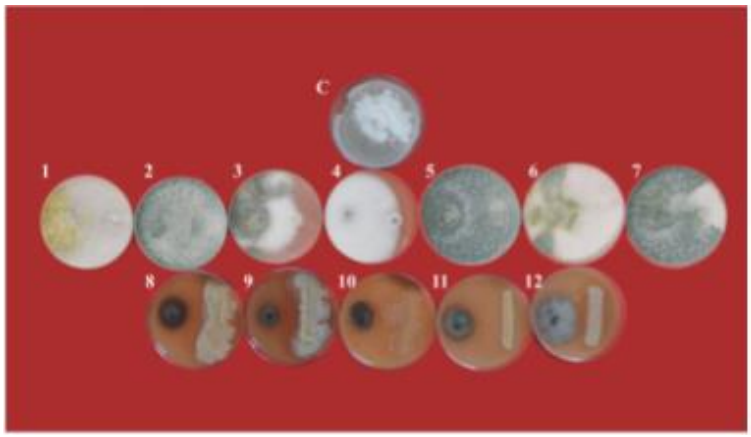
S. No.	Biocontrol agents	Per cent inhibition of mycelial growth
1.	*Trichoderma koningiopsis* (TNAU)	77.11 (61.42)*
2.	*Trichoderma harzianum* (IOF, Dharwad)	77.91 (61.97)
3.	*Trichoderma viride* (Multiplex Nisarga)	87.55 (69.34)
4.	*Trichoderma harzianum* (Kalpavruksha)	81.93 (64.84)
5.	*Trichoderma asperillium* isolate 07 (UASB)	77.91 61.97)
6.	*Trichoderma harzianum* isolate 109 (UASB)	73.90 (59.27)
7.	*Trichoderma harzianum* isolate 69 (UASB)	83.53 (66.06)
8.	*Bacillus subtilis* (UASR)	63.86 (53.04)
9.	*Bacillus amyloliquifaciens* (TNAU)	76.31 (60.87)
10.	*Bacillus subtilis* (IOF,Dharwad)	61.85 (51.85)
11.	*Pseudomonas fluorescens* (UASR)	57.83 (49.51)
12.	*Pseudomonas fluorescens* (IOF,Dharwad)	51.81 (46.04)
S.Em. ±		0.67
C.D. @1%		2.66
C.V.		1.98

***** Arc sine transformed values

Table 3: *In vitro* evaluation of different biocontrol agents against *Fusarium oxysporum*

S. No.	Biocontrol agents	***F.oxysporum***
1.	*Trichoderma koningiopsis* (TNAU)	71.43 (57.69)
2.	*Trichoderma harzianum* (IOF,Dharwad)	64.68 (53.54)
3.	*Trichoderma viride* (Multiplex Nisarga)	67.86 (55.46)
4.	*Trichoderma harzianum* (Kalpavruksha)	61.90 (51.89)
5.	*Trichoderma asperillium* isolate 07 (UASB)	67.86 (55.46)
6.	*Trichoderma harzianum* isolate 109 (UASB)	60.32 (50.95)
7.	*Trichoderma harzianum* isolate 69 (UASB)	64.29 (53.30)
8.	*Bacillus subtilis* (UASR)	47.22 (43.41)
9.	*Bacillus amyloliquifaciens* (TNAU)	60.71(51.19)
10.	*Bacillus subtilis* (IOF,Dharwad)	57.14 (49.11)
11.	*Pseudomonas fluorescens* (UASR)	42.06 (40.43)
12.	*Pseudomonas fluorescens* (IOF,Dharwad)	36.51 (37.17)
S.Em. ±		1.81
C.D. @1%		2.46
C.V.		2.15

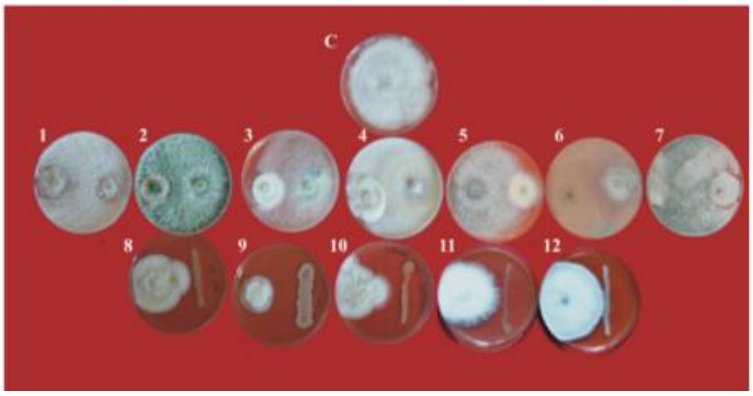
***** Arc sine transformed values



1. *Trichoderma koningiopsis* (TNAU)
2. *Trichoderma harzianum* (IOF, Dharwad)
3. *Trichoderma viride* (Multiplex Nisarga)
4. *Trichoderma harzianum* (Kalpavruksha)
5. *Trichoderma asperillium* isolate 07 (UASB)
6. *Trichoderma harzianum* isolate 109 (UASB)
7. *Trichoderma harzianum* isolate 69 (UASB)
8. *Bacillus subtilis* (UASR)
9. *Bacillus amyloliquifaciens* (TNAU)
10. *Bacillus subtilis* (IOF, Dharwad)
11. *Pseudomonas fluorescens* (UASR)
12. *Pseudomonas fluorescens* (IOF, Dharwad)

C-Control

Plate 1: *In vitro* evaluation of bio-agents against *C. fimbriata*



1. *Trichoderma koningiopsis* (TNAU)
2. *Trichoderma harzianum* (IOF, Dharwad)
3. *Trichoderma viride* (Multiplex Nisarga)
4. *Trichoderma harzianum* (Kalpavruksha)
5. *Trichoderma asperillium* isolate 07 (UASB)
6. *Trichoderma harzianum* isolate 109 (UASB)
7. *Trichoderma harzianum* isolate 69 (UASB)
8. *Bacillus subtilis* (UASR)
9. *Bacillus amyloliquifaciens* (TNAU)
10. *Bacillus subtilis* (IOF, Dharwad)
11. *Pseudomonas fluorescens* (UASR)
12. *Pseudomonas fluorescens* (IOF, Dharwad)

C-Control

Plate 1: *In vitro* evaluation of bioagents against *F. oxysporum*

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