



IN VITRO EFFICACY OF FUNGAL AND BACTERIAL ANTAGONISTS AGAINST *FUSARIUM OXYSPORUM* F. SP. *CEPAE* CAUSING BASAL ROT OF ONION

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Abstract

Basal rot of onion is one of the most destructive disease, caused by *Fusarium oxysporum* f. sp. *cepae*. Efficacy of various biocontrol agents was evaluated for the potential to manage the basal rot of onion *in vitro*. Among the tested isolates of *Trichoderma viride* (Tv 5) gave the greatest (82.86%) inhibition and *Pseudomonas fluorescens* (Pf2) exerted significantly the greatest (80.82%) reduction of mycelial growth of *F. oxysporum* f. sp. *cepae*. Rest of the bioagents tested also caused significant mycelial inhibition of the test pathogen. ITS-PCR amplification of all the 10 isolates with primers ITS3 and ITS4 resulted in an amplicon of 250 bp in size indicating the isolates to be *Fusarium* spp.

Key words: Onion, Basal rot, *T. viride* and *P. fluorescens*

Introduction

Onion (*Allium cepa* var. *aggregatum* G. Don) is one of the important crops grown in India amongst vegetables and spices. Onion rich in flavanoids like quercetin and sulfur compounds, such as allyl propyl disulphide have been perceived benefits to human health. Basal rot caused by *Fusarium oxysporum* f. sp. *cepae* is the most destructive disease of onion in all growing areas of the world and causes severe losses in the productivity both in field and in storage condition (Coskuntuna and Ozer, 2008; Lager, 2011). Basal rot of onion causes more than 50% of yield loss (Mishra *et al.*, 2014). Losses due to this disease ranging from 30 % to 100 % (Anupama *et al.*, 2014; Priya *et al.*, 2016). The recent investigations have focused on biological control and induced resistance (Dias-Arieira *et al.*, 2012) in combination for the management of this pathogen. Accordingly, the present study was conducted to evaluate the efficacy of *T. viride* and *P. fluorescens* against *F. oxysporum* f. sp. *cepae* both *in vitro* conditions.

Materials and Methods

Isolation of *F. oxysporum* f. sp. *cepae*

The pathogen was isolated from the infected bulbs of onion by tissue segment method (Rangaswami, 1958). The infected portions of bulb were cut into small pieces using sterilized scalpel and these were surface sterilized with 1.0 per cent sodium hypo chloride for one minute and washed in three changes of sterile distilled water and then placed on Petri dish containing Potato Dextrose Agar (PDA) medium. These plates were incubated at room temperature ($28 \pm 2^\circ\text{C}$) for five days and observed for the growth of fungus. The hyphal tips of fungi grown from the plates were transferred aseptically to PDA slants for maintenance of the culture. The pathogens were identified based on their cultural and morphological characters.

Molecular characterization of *F. oxysporum* f. sp. *cepae*

DNA Extraction

All the above isolates were grown in 100ml of Potato dextrose broth medium (PDA without agar) for 9 days in

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a BOD incubator at $27 \pm 1^\circ\text{C}$, 65 % RH and a 16:8 h photoperiod. The genomic DNA was extracted and purified using the CTAB buffer method modified from Nicholson *et al.* (1996). The mycelial culture was frozen and ground in liquid nitrogen into fine powder and an aliquot (1 ml) of CTAB (hexadecyltrimethyl ammonium bromide) buffer (CTAB 22 mM, sarkosyl 134 mM, sorbitol 137 mM, EDTA 22 mM, poly vinyl poly pyrrolidone (PVPP) 1%, NaCl 1.2 mM) was added. The resultant mixture was ground into paste in the same mortar. The ground sample was extracted in CTAB buffer for 60 min at 65°C . One third volume of 5M potassium acetate was added along with 1 ml chloroform: isoamyl alcohol (24:1), mixed and held at 20°C for 20 min. the mixture was then centrifuged at 1900 rpm for 15 min and the aqueous phase was added to two volumes of 95% ethanol. The DNA was precipitated at 850 rpm and the pellet was washed twice with 70% (v/v) ethanol before being dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). Extracted DNA was electrophoresed in 0.8% agarose gel at 50 mA for 90 min. the gels were stained for 15 min in ethidium bromide (EtBr 0.5 mg ml⁻¹) and destained for 15 min in distilled water; alternatively, EtBr was incorporated directly into gel at a rate of 0.5 mg ml⁻¹. The gel was photographed using UV trans-illuminator gel documentation system and DNA concentrations were estimated spectrophotometrically using nano drop ND-1000.

PCR amplification

PCR analysis was carried out to amplify the internal transcribed spacer (ITS) region in the DNA of the *Fusarium* isolates. The forward primer of *ITSF3* was 5'-GCATCGATGAAGAACGCAGC-3' and the reverse of *ITSR4* was 5' TCCTCCGCTTATTGATATGC-3' (White *et al.* 1990). The PCR reaction mixture (20 µl) contained 0.3 U *Taq* DNA polymerase, IX assay buffer (10 mM pH 9.0 TRIS- HCl, 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin), 150 µM of each dNTP, 1 µl of each forward and reverse primer at a final concentration of 0.25 µM and 60 ng template DNA. The reaction mix without template DNA was used as water blank. The PCR reaction profile was composed of 35 cycles, with strand separation at 90°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min. The program was extended for 10 min at 72°C . The amplification products were analyzed on 1.0% agarose- EtBr gel.

ITS - PCR analysis

The molecular size of each fragment was estimated using standard curve of migration versus the log of the molecular size of 100 bp ladder. Each fragment was scored on the basis of the presence or absence of

particular fragments.

Isolation of native antagonists from rhizosphere soil

Trichoderma spp.

Rhizosphere soil samples collected from ten different locations were used for the isolation of *Trichoderma* isolates by soil dilution plating technique using *Trichoderma* selective medium (TSM) (Elad and Chet, 1983). These *Trichoderma* cultures were purified by single hyphal tip method and used for the studies. *Trichoderma* spp., thus isolated was subjected for identification based on the key suggested by Domsch *et al.* (1980). On the whole ten isolates of *Trichoderma* spp. were isolated. These isolates were designated as Tv₁-Tv₁₀.

Isolation of native antagonistic bacteria

Antagonistic bacteria were isolated from the rhizosphere soil collected from different onion growing areas of Tamilnadu by serial dilution method on King's B medium, incubating at room temperature for 24 h. Colonies with characteristics of *Pseudomonas* sp., were isolated individually and purified by streaking them on King's B medium. For the identification of *Pseudomonas* sp. isolates, certain biochemical tests were conducted according to Bergey's Manual for Determinative Bacteriology (Breed *et al.*, 1989). The identified isolates were designated as Pf₁-Pf₁₀.

Screening of the fungal and bacterial biocontrol agents against *F. oxysporum* f. *cepae* *in vitro*

The antagonistic activity of bio control agents against *F. oxysporum* f. *cepae* was tested by dual culture technique (Dennis and Webster, 1971). At one end of the sterile Petri dish containing 15 ml of sterilized and solidified PDA medium a 9 mm mycelial disc obtained from five day old culture of *Trichoderma* spp. was placed under aseptic conditions. Similarly, at the opposite end approximately 75 mm away from the *Trichoderma* culture disc, a 9 mm culture disc of *F. oxysporum* f. *cepae* was placed and incubated. A control was maintained by inoculating *F. oxysporum* f. *cepae* alone at one end of the Petri dish. The plates were incubated at room temperature ($28 \pm 2^\circ\text{C}$) for three days. In case of *P. fluorescens* one cm long streak was gently made onto the medium using two days old culture. The radial growth (in mm) of the pathogen and the test antagonists and the extent of the inhibition zones (in mm) developed between the two colonies were measured. The effective antagonists were identified based on the inhibition of the growth of the pathogen. The radial mycelial growth of the pathogen and per cent reduction over control was calculated by using the formula (Vincent, 1927).

Per cent inhibition (I) = $C-T/C \times 100$

Where, C- mycelial growth of pathogen in control

T- Mycelial growth of pathogen in dual plate

I- inhibition per cent

Result and Discussion

Molecular characterization

In this present study the results obtained from PCR amplification of ITS + 5.8s region with primer pair of *ITSF3* and *ITSR4* produced a gene product of 200- 250 bp confirming the isolates to be *Fusarium* sp. Further studies are needed to confirm the interstrain variation (Fig. 1).

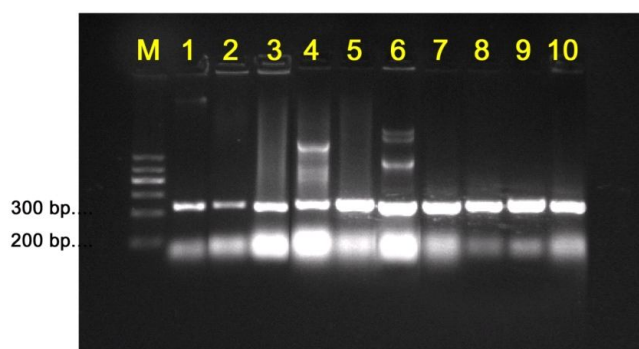


Fig.1: PCR amplification of *Fusarium* sp.

Molecular characteristic of *F. oxysporum* f. sp. *cepae*

In this study, we selected ITS- 3.8s- ITS 4 region for amplification using previously reported primer *ITSF3* and *ITSR4* (Girija Ganeshan *et al.*, 2009) and we obtained approximately 200-250 bp common fragments in all isolates of *Fusarium*. Kamel *et al.* (2003) reported that using primers ITS-Fu-f and ITS-Fu-r, specifically amplified a 398 bp fragment from all *Fusarium* species isolates. Girija Ganeshan *et al.* (2009) reported that amplified product of *F. oxysporum* f. sp. *cepae* isolate was digested into *AluI* and obtained about 496 and 245bp. Mishra *et al.* (2014) reported that using (ITS1F/ITS1R) primer *Fusarium* isolates were amplified and obtained a gene product with a size of 230 bp of *Fusarium* sp. and 280 bp for *Fop* isolates. Kumar *et al.* (2016) showed that ITS-PCR of 11 representative isolates of *F. mangiferae* using ITS1 and ITS4 primer amplified a common 570 bp band among all isolates. These earlier reports corroborates with the present findings.

Antifungal activity of *Trichoderma* sp. against mycelial growth of *F. oxysporum* f. sp. *cepae* in *in vitro* (Dual culture technique)

In general all the native *Trichoderma* spp. tested significantly inhibited the mycelial growth of *F. oxysporum*

f. sp. *cepae* (Table 1). However, among the isolates, the isolate Tv₅, showed the maximum inhibition and significantly inhibited the growth of *F. oxysporum* f. sp. *cepae* (15.42 mm), which was 82.86 per cent reduction on the growth of the pathogen when compared to control. This was followed by the isolates Tv₃ and Tv₉ in the decreasing order of merit, which inhibited the growth of *F. oxysporum* f. sp. *cepae* by 81.98 and 79.86 per cent over control. The least growth inhibition of the pathogen (44.17%) was exhibited by the isolate Tv₇.

The results of the present study correspond with Ilhe (2013) who observed that *T. viridewas* found to be most effective in inhibiting the growth of *F. oxysporum* f. sp. *cepae*. Hacer Handan and Oktay (2015) observed that *T. harzianum* 16 and 23 strains showed significant inhibition of mycelial growth of the pathogenic strains of *F. oxysporum*. Both *T. harzianum* strains produced volatile and non-volatile metabolites that inhibited growth of *F. oxysporum* strains. Narayan Prasad Verma *et al.* (2018) showed variation in the antagonistic activities of *Trichoderma* sp. isolates against the tested *Fusarium* sp. and also, inhibition of the pathogen may be attributed to the production of secondary metabolites (such as glioviridin, viridin and gliotoxin) the antagonists (Inbar *et al.*, 1994). Several studies (Jayalakshmi *et al.*, 2009; Muhammad and Amusa, 2003; Shabir *et al.*, 2013) reported that inhibition of some soil borne pathogens, including *Fusarium* spp. by *Trichoderma* species could probably be due to the secretion of extracellular cell wall degrading enzymes such as chitinase, β -1, 3- glucanase, β -1, 6-glucanase, protease, cellulase and lectin, which

Table 2: Antifungal activity of *Pseudomonas fluorescens* against mycelia growth of *F.oxysporum* f. sp. *cepae* in *in vitro* (Dual culture technique)

S. No.	Isolates	Mycelial growth of <i>F.oxysporum</i> f. sp. <i>cepae</i> (mm)	Percent (%) inhibition over control
1.	Pf ₁	35.73 ^d	60.10
2.	Pf ₂	23.32 ^a	80.82
3.	Pf ₃	48.55 ⁱ	46.05
4.	Pf ₄	29.72 ^b	78.22
5.	Pf ₅	49.62 ^j	44.86
6.	Pf ₆	41.81 ^f	53.54
7.	Pf ₇	31.75 ^c	64.72
8.	Pf ₈	38.98 ^e	56.68
9.	Pf ₉	46.12 ^h	48.75
10.	Pf ₁₀	42.35 ^g	52.94
11.	Control	90.00	-

*Mean of three replications

*In a column, means followed by a common letter are not significantly different at 5% level by Duncan's multiple range test (DMRT)

Table 1: Antifungal activity of *Trichoderma viride* against mycelial growth of *F. oxysporum* f. sp. *cepae* in vitro (Dual culture technique)

S. No.	Isolates	Mycelial growth of <i>F.oxysporum</i> f. sp. <i>cepae</i> (mm)	Per cent (%) inhibition over control
1.	Tv ₁	35.78 ^g	60.24
2.	Tv ₂	47.12 ⁱ	47.64
3.	Tv ₃	16.21 ^b	81.98
4.	Tv ₄	38.21 ^h	57.54
5.	Tv ₅	15.42 ^a	82.86
6.	Tv ₆	23.72 ^e	73.64
7.	Tv ₇	50.24 ^j	44.17
8.	Tv ₈	28.82 ^f	67.97
9.	Tv ₉	18.12 ^c	79.86
10.	Tv ₁₀	20.21 ^d	77.54
11.	Control	90.00 ^k	-

*Mean of three replications

*In a column, means followed by a common letter are not significantly different at 5% level by Duncan's multiple range test (DMRT)

help mycoparasites to colonize their host.

Efficacy of native bacterial isolates against *F. oxysporum* f. sp. *cepae* (Dual culture)

The results presented in table 2 revealed varying degree of antagonism by the isolate of *Pseudomonas* against *F. oxysporum* f. sp. *cepae*. Among the *Pseudomonas* isolates, Pf₂ produced significantly the minimum mycelial growth (23.32 mm) accounting for 80.82 per cent reduction on the mycelial growth of *F. oxysporum* f. sp. *cepae* over control. This was followed by isolate Pf₄ which recorded 78.22 per cent reduction on the mycelial growth over control. The isolate Pf₁₀ was the least effective among *Pseudomonas* isolates as it recorded the minimum percent inhibition control.

Boukerma *et al.* (2017) reported that the potential of *P. fluorescens* PF15 and *P. putida* PP27 showed significant inhibition of the *F. oxysporum* f. sp. *lycopersici* in tomato. Malathi (2015) reported that, Pf 12 and Pf 27 of the *Pseudomonas* isolates were found to be the most effective in inhibiting the growth of *F. oxysporum* f. sp. *cepae*. Many strains of *Pseudomonas* have been found to produce broad spectrum antibiotics including phenazine, pyrrolnitrin, pyoverdine and 2,4 diacetylphloroglucinol (Gardener *et al.*, 2000), lytic enzymes such as chitinases and β -1, 3-glucanases which degrade fungal chitin (Velazhahan *et al.*, 1999), siderophore (Loper, 1988), HCN (Ahl *et al.*, 1986) and induced systemic resistance (Van Peer *et al.*, 1991). Several earlier workers have suggested a significant role of secondary metabolites such as antibiotics, siderophores

of pseudomonads in suppression of fungal pathogens (Vinodkumar *et al.*, 2007; Sreedevi and Charitha Devi, 2012). Such multiplicity of mechanisms exerted by *P. fluorescens* might be attributed as the reason for the reduction in the growth of the pathogen.

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