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Research Article

Identification of antifungal proteins from fungal and bacterial antagonists against *Colletotrichum falcatum* causing sugarcane red rot.

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ABSTRACT: Effective fungal and bacterial antagonists having hyperparasitism and antibiosis mode of antagonistic activity against *Colletotrichum falcatum* were selected based on the suppression of pathogen growth and production of cell wall lytic enzyme chitinase. The effective strains were utilized to identify the sources of antifungal proteins suppressing enzymes and toxin, the pathogenicity determinants of *Colletotrichum falcatum* during their interaction. The effective biocontrol agents identified against *C. falcatum* inhibited the production of extracellular enzymes/ proteins of the pathogen and also detoxified/ inactivated the toxin/ enzymes, when the antagonists were grown along with pathogen or its metabolites in liquid cultures as well as at the point of entry, where it failed to elicit characteristic symptoms on the host. Final outcome of the study resulted in identification of high molecular proteins specifically produced by the antagonists to inactivate the pathogenic metabolites in SDS–PAGE.

KEY WORDS: Sugarcane, *Colletotrichum falcatum*, secondary metabolites, antifungal proteins.

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INTRODUCTION

Sugarcane is one of the most important cash crops grown in India due to its adaptability to be cultivated under a wide range of climate, cultural and soil conditions. The crop is infected by fungi, bacteria and viruses. Among the fungal diseases, red rot caused by the fungus *Colletotrichum falcatum* Went is the major constraint in the production of the crop (Viswanathan, 2010). A disease is said to be set–in when a compatible interaction between pathogen and its host is established. The nature of subsequent biological impairments caused by the pathogen largely depends on a variety of biochemical blue prints encompassing production of lytic enzymes and/or toxins by the pathogen (Panopolous and Peet, 1985). Enzymes secreted by pathogens cause dissolution of plant cell wall barrier, making the protoplasm vulnerable to attack by toxins which tear apart the naked protoplasm with an eventual loss of electrolytes and ultimate death of the cell. Pathogenic variability in *C. falcatum* has been well established with the production of various metabolites *viz.*, toxin, enzymes and melanin and their production was positively correlated with the symptom expression in the host (Malathi *et al.*, 2010).

An integrated approach involving different management practices like breeding for disease resistance, cultural practices, chemical and biological control and the biotechnological approach for the development of transgenic plants with disease resistance antifungal genes have been suggested for control of *C. falcatum*. Biocontrol in combination with molecular techniques involving both fungal and bacterial antagonists are being studied for the possible protection of sugarcane against red rot pathogen. Strains of pseudomonads and *Trichoderma* were shown to be effective against the pathogen both *in vitro* and *in vivo* conditions (Viswanathan and Samiyappan, 1999; Malathi *et al.*, 2008). Work on isolation and characterization of antifungal genes for cell wall lytic enzymes *viz*. chitinases and β, 1-3, glucanase from these biocontrol agents was carried out at the SBI (Viswanathan, *et al*., 2003). In this context, an attempt has been made to study the capability of antagonists for the possible inhibition/ inactivation of important pathogenic metabolites *viz.,* toxin and enzymes. Studies by Malathi *et al.* (2002a) clearly demonstrated that inactivated pathogen toxin failed to elicit characteristic symptoms on the host. In the present investigation antifungal proteins responsible for suppressing the pathogenicity has been elucidated by SDS–PAGE.

MATERIALS AND METHODS

Pathogen

The virulent isolate of *C. falcatum* (Cf671) was obtained from type culture collections of the plant pathology section of the Sugarcane Breeding Institute and maintained in oat meal agar.

Antagonistic strains

Fungal (*Trichoderma* spp.) and bacterial (Fluorescent pseudomonads) antagonistic strains isolated from sugarcane rhizosphere (Viswanathan and Samiyappan, 2000; Nallathambi *et al.,* 2000) were employed in the study. Isolates of *Trichoderma* spp. include *T. harzianum* –T5 & T62, *T. viride* –T33, T48 & T62 and fluorescent pseudomonads include *P. fluorescens* – ARR–1G, ARR–2, ARR–10, FP–7, VPT-4 & VPT–10. Fungal cultures were maintained on oat meal agar and bacterial cultures were maintained on King's B media, respectively.

Production of chitinase and chitinolytic activity

Both fungal and bacterial antagonistic strains were grown in minimal medium (K_2HPO_4 – 1.5g; $NaNO_3$ – 2g; $MgSO_4$ –1.4g; Distilled water – 1000ml) alone and amended with colloidal chitin (0.1%). The pseudomonad strains were grown for 48h, while, *Trichoderma* strains were grown for 120h in both the media and cell free culture filtrate was obtained by centrifugation (17,226g, 4°C). The filtrate was used for assessing the chitinolytic activity to determine their antibiosis mode of action.

Chitinase activity of antagonistic strains was assessed on the basis of clearance seen on the colloidal chitin agar medium (Hsu and Lockwood, 1975). On hundred µl of cell free culture filtrate of both fungal and bacterial antagonistic strains grown on chitin amended medium was placed in 8 mm diameter wells in water agar medium incorporated with 0.2% colloidal chitin and incubated at room temperature (25-30°C). Observations on clearing zone around the treated wells were recorded 4 days later. Culture filtrate from each strain was treated similarly in three wells and suitable checks were maintained using the broth as such instead of culture filtrate from antagonistic strains.

Microbial antagonism

The antagonistic activity of microbial strains against *C. falcatum* was studied by dual culture technique on oat meal agar in the presence or absence of chitin. Antagonism of *Trichoderma* spp. was tested by keeping 9 mm disc of pathogen mycelium at one end of seeded oats agar with or without colloidal chitin at 0.2% and 9 mm disc of *Trichoderma* was placed 2 days after the pathogen inoculation at the opposite end. For testing bacterial antagonists, the pathogen disc was placed at the centre and 24-48h old bacterial culture was streaked on three sides at 2 cm away from the edge of the growing mycelium. In both the cases the mycelial inhibition was observed till the pathogen attained full growth in petridishes as check and expressed in percent mycelial inhibition.

Effect of antagonists on *C. falcatum* secondary metabolites

Based on above studies, efficient strains of *P. fluorescens*- Pf (VPT4 & FP7) and *T. harzianum*- Th (T5 and T62) were selected for studying their efficacy against *C. falcatum* metabolites. As a preliminary attempt, *T. harzianum* strains were employed for all the studies and *P. fluorescens* was tried only on degradation of partially purified toxin as mentioned below. Efficacy of antagonists during host pathogen interaction has been demonstrated by the leaf bioassay technique developed at the Institute (Malathi *et al*., 2006), which involves pinpricking of 1cm diameter in the middle of 15cm size young unfolded leaves and placing the spore suspension over the pinpricked spots. After stipulated incubation period, the droplets were harvested and subjected for production of enzymes by the pathogen or antagonist or their mixture. *T. harzianum* spores were inoculated along with *C. falcatum* conidial suspension and the droplets were analysed for the production of pectinolytic (Endo & Ecto PG) and cellulolytic (Cx) enzymes (Kapat *et al.,* 1998).

For molecular studies on microbial degradation of enzymes, the *T. harzianum* (T5) and *C. falcatum* were grown individually or in combination in the specific medium for enzymes. Simultaneously, the *T. harzianum* isolate was grown on 7 days old *C. falcatum* culture filtrate from the same specific medium. Hence, the treatments included were (1) Simultaneous addition of pathogen and the *Trichoderma* spores (2) Addition of *Trichoderma* spores 4 days after the pathogen inoculation (3) Growing of *Trichoderma* completely on 100% pathogen filtrate. (4) Growing of *C. falcatum* and *Trichoderma* individually on specific medium broth. Here the specific media used were pectin medium for pectinolytic enzymes and toxin production medium for enzymes involved in toxin production. The pectin medium used was 1.5% pectin amended Richard's broth (Kapat *et al.,* 1998) and the toxin production medium was host extract amended Czapeck's broth (Mohanraj *et al*., 2002). The host extract was prepared by crushing the peeled sugarcane top 3 nodes and it was added @ extract from 250g of tissue to compensate the 3% sucrose in the Czapeck's broth. In all the conditions the pathogen and antagonist were grown for 7 days and the extracellular proteins were purified by ammonium sulphate precipitation and dialysis.

To study the inhibitory effect of *P. fluorescens* strains on partially purified toxin, the cultures were grown on toxin amended Czapeck's broth for 4 days (Malathi *et al*., 2002a) and the extracellular proteins were purified. The purified proteins were analysed by SDS-PAGE. The partially purified toxin was obtained by growing the pathogen in toxin production medium and following the protocol developed at the Institute (Mohanraj *et al*., 2002).

RESULTS AND DISCUSSION

Antibiosis mode of antagonists

Chitinases are widely distributed in nature and play an important role in hydrolysis of β 1-4 linkages of GlcNAc polymer chitin, a structural polysaccharide present in different organisms, mainly, arthropods and fungi (Cabib, 1987). Since all the antagonistic strains were able to produce chitinase in the medium, their antibiosis activity was proved in terms of lytic zone in the chitin-amended medium. The zone of lysis was calculated by deducting the diameter of the well from the total width of the zone and the maximum lytic zone (more than 10mm) was produced by ARR1G, FP7 and VPT4 in pseudomonads and T5 and T62 in *T. harzianum*. It was inferred that there was considerable difference between strains in the development of zone around the well. It supports the results on chitinase production and chitinolytic activity of selected antagonistic strains (Viswanathan *et al.* 2003, Viswanathan and Samiyappan, 2001)

Table 1. Inhibitory effect of antagonistic strains on *Colletotrichum falcatum* growth

Antagonistic strains	Per cent mycelial inhibition		
	Without chitin	With chitin	Mean
*Pseudomonas fluorescen*s strains			
ARR1G	53.9 (46.34)	66.7 (54.34)	60.30 (50.34)
ARR2	24.4 (29.33)	27.2 (31.52)	25.80 (30.43)
ARR10	24.4 (29.33)	27.2 (31.52)	25.80 (30.43)
FP7	45.6 (42.32)	46.7 (43.28)	46.15 (42.80)
VPT4	61.1 (51.55)	71.7 (57.63)	66.40 (54.59)
VPT10	0.0 (4.05)	0.0 (4.05)	0.0 (4.05)
Trichoderma spp.			
T5	59.5 (50.19)	63.6** (52.93)	61.55 (51.56)
T33	57.2 (48.83)	58.3* (49.80)	57.75 (49.31)
T48	52.8 (46.53)	53.4* (46.53)	53.10 (46.53)
T52	55.6 (48.06)	56.6* (48.64)	56.10 (48.35)
T62	55.6 (48.64)	62.2** (52.93)	58.90 (50.79)
Mean	47.49 (42.21)	52.85 (45.65)	50.17 (43.93)

(P = 0.05)	Media	0.35
	Antagonitic Strains	0.82
	Interaction	1.15

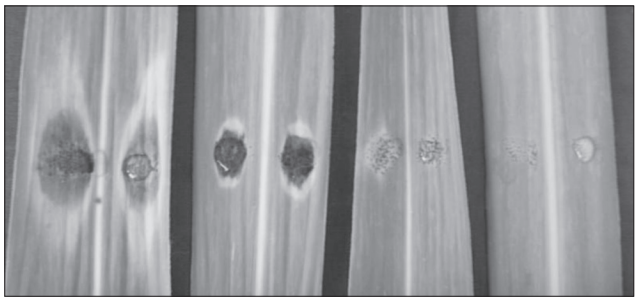
** Hyperparasitism with reduced mycelial density
* Hyperparasitism alone

Inhibitory effect of antagonistic strains against *C. falcatum* growth

Antagonistic activity of both fungal and bacterial strains was tested under the influence of chitin in oat meal agar medium. Results postulated that almost all the strains were able to inhibit the mycelial growth in the presence or absence of the chitin (Table 1). However, addition of chitin resulted in the enhanced inhibition of mycelial growth by ARR 1G, FP7 and VPT4 in *P. fluorescens* and in all the strains of *Trichoderma* spp. It was interesting to note that the degree of hyperparasitism by *Trichoderma* strains was highly influenced by the chitin. Since, the effect of chitin was proven in the study, it can be directly correlated with the influence of chitinase during antagonism of *T. harzianum* (Sivan and Chet, 1989; Cruz *et al*., 1992). There was delay in the onset and intensity of sporulation of the pathogen by the addition of all the antagonistic strains. Of all the strains least antagonism was given by the pseudomonad strain VPT 10. Influence of chitin/ cell wall on extracellular enzymes of antagonistic strains to inhibit the *C. falcatum* conidial germination, germ tube elongation and mycelial growth were proven by inhibition zone technique (Viswanathan *et al*., 2003). Present investigation clearly demonstrated the hyperparasitism and antibiosis mode of fungal and bacterial antagonistic strains respectively, and based on which effective strains were selected for the identification of antifungal proteins. *In vitro* and *in vivo* efficacy of various fungal and bacterial antagonists have been well demonstrated against *C. falcatum* for field application either individually or in combination with fungicides by proving their efficacy on various pathotypes of the pathogen (Malathi *et al*., 2002b; 2008).

Inhibitory effect of *T. harzianum on C. falcatum* enzyme production

On leaf bioassay, *T. harzianum* inhibited the pathogen conidial germination and germ tube elongation depending on the time of inoculation with the pathogen. Correspondingly, there was a significant reduction in the production of cell wall lytic enzymes (pectinolytic & cellulolytic) (Fig. 2)



C. falcatum | *C. falcatum* + *T. harzianum* | *T. harzianum* | Distilled Water

Fig. 1. Interaction between *Colletotrichum = falcatum* and *Trichoderma harzianum* on sugarcane leaf

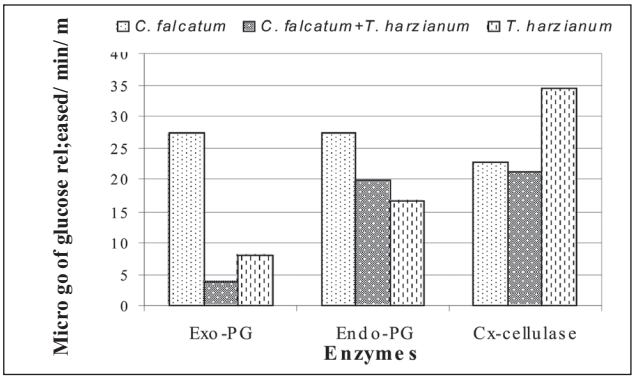


Fig. 2. Enzyme production during interaction of sugarcane - host, *Colletotrichum falcatum* and *Trichoderma harzianum*

and symptoms as compared to pathogen alone (Fig. 1). The inhibitory proteins of *T. harzianum* were characterized by SDS-PAGE analysis which showed the induction of specific proteins by the *T. harzianum* in pectin medium (45-50 kDa) against pectinolytic enzymes of the pathogen inoculated as such or its filtrate (Fig. 3). Induction of high molecular

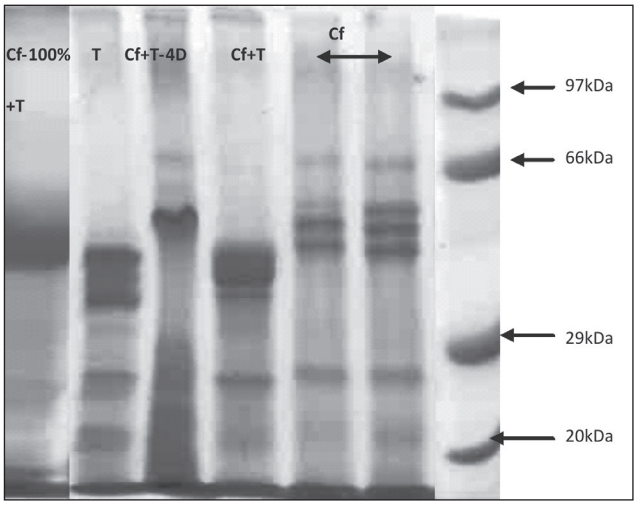


Fig. 3. Microbial degradation of pectinolytic enzymes of *Colletotrichum falcatum* by *Trichoderma harzianum*

C f - *C. falcatum* alone in pectin medium; T - *T. harzianum* alone in pectin medium
Cf+T – *C. facatum* & *T. harzianum* were inoculated at the same time
Cf+T – *T. harzianum* was inoculated 4days after the inoculation of *C. falcatum*
Cf-100%+T – *T. harzianum* grown in 100% culture filtrate of *C. falcatum* in pectin medium

weight protein (110 kDa) by both the *T. harzianum* strains grown along with the pathogen simultaneously in toxin production medium was observed. It is interesting to note that there was specific induction of protein during the interaction in both the media and such proteins do not have any similarity with the pathogen or *Trichoderma* proteins in the same media. Results of the present study clearly demonstrated the interference of antagonists in hydrolytic enzyme production or their inhibitory effect on degradation of extracellular enzymes produced by *C. falcatum*. This was proved earlier in *Botrytis cinerea* by *T. harzianum* (Zimand *et al*., 1996; Kapat *et al*., 1998). Recently, several groups started using interactive proteome studies to reveal the changes during microbial and/ host interactions (Woo *et al*., 2006 and Marra *et al.,* 2006). They used two-dimensional (2-D) electrophoresis to analyze separately collected proteomes from each single, two- or three-partner interaction (i.e., plant, pathogenic and antagonistic fungus alone and in all possible combinations) and characterized differentially expressed proteins.

Toxin inactivation / degradation by *T. harzianum*

In another study, growth of *P. fluorescens* in toxin amended medium detoxified/inactivated the partially purified toxin of *C. falcatum* by producing a 97 kDa high molecular protein (Fig. 4) and the treated toxin was incapable of producing symptoms in the host. Reduction in

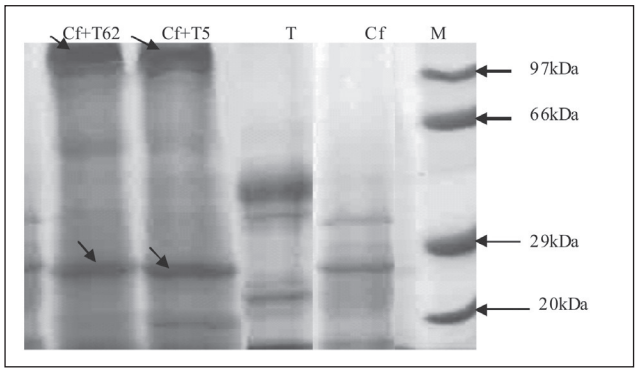


Fig 4. Microbial degradation in toxin basal medium

C f - *C. falcatum* alone in toxin production medium
T - *T. harzianum* T5 alone in toxin production medium
Cf+T5 &T62 – *T. harzianum* strains grown on 100% culture filtrate of *C. falcatum* obtained from toxin production medium

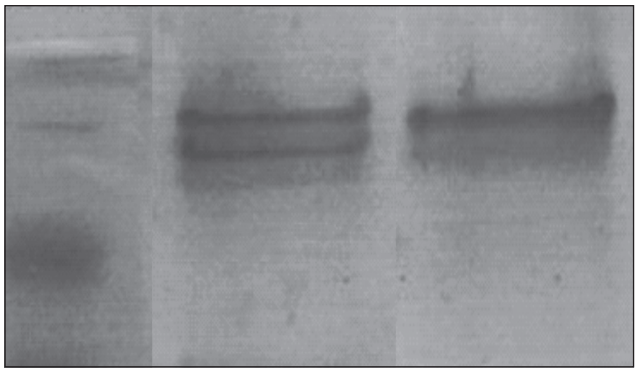


Fig. 5. Extracellular protein profile of *Pseudomonas fluorescens* grown on *Colletotrichum falcatum* toxin amended medium

Lane 1 – Protein marker (29 to 205kDa - Genei)
Lane 2&3 – Extracellular protein profile of *P. fluorescens* strains grown on toxin amended medium

symptom production was reported with various strains of *P*. *fluorescens* in the earlier study by Malathi *et al*. (2002). Detoxification or inactivation of the phytotoxin reduces the toxicity of metabolite produced by plant pathogens which has been clearly shown by leaf bioassay (Results not shown). Microorganisms form an exotic source of enzymes which are capable of inactivating synthetic chemicals that are potentially phytotoxic. Reduction in the symptom production by the treated toxin has already been proved in our preliminary studies with the microbial detoxification of *C. falcatum* toxin (Malathi *et al*., 2002a), while, the induction of high molecular protein from pseudomonad strains has been demonstrated by SDS-PAGE. Sriram *et al*. (2000) reported that *Trichoderma viride* inactivated the phytotoxin of *Rhizoctonia solani* by the production of high molecular mass protein of 110 kDa. Further characterization of these specific proteins and their validation by interactive proteomics will be helpful for transgenic approach as reported in earlier studies. In sugarcane, expression of *AlbD* (albicidin hydrolase) in transformed *X. albilineans* strains abolished the capacity to release albicidin toxins and to incite leaf scald disease symptoms in sugarcane. The gene is a promising candidate for transfer into sugarcane to confer disease resistance (Zang *et al*., 1999). Melanization of appressoria is essential for the penetration into the host by fungal species like *Magnoporthe* sp. and *Colletotrichum* sp. Alfastatin A (AsA) isolated from the mycelia of *Streptomyces* sp. completely inhibits the melanin synthesis of *C*. *lagenarium* by interfering the enzymes involved in melanin biosynthesis (Okamoto *et al*., 2001). Above results indicated that the present study lays base for identification and isolation of specific antifungal protein genes from efficient microbial strains targeted on pathogenic weapons which would lead to applicability for in–built resistance against the red rot pathogen in future.

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