Chitinase Expression Due to Reduction in Fusaric Acid Level in an Antagonistic *Trichoderma harzianum* S17TH

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Abstract To study the effect of reduction in phytotoxin level on fungal chitinases, antagonistic *Trichoderma* spp. were screened for their ability to reduce the level of fusaric acid (FA), the phytotoxin produced by *Fusarium* spp. A *T. harzianum* isolate S17TH was able to tolerate high levels of FA (up to 500 ppm) but was unable to reduce the toxin to a significant level (non-toxic) added to minimal synthetic broth (MSB). However, the isolate was able to reduce 400 ppm FA in the liquid medium after 7 days to a non-toxic level and displayed similar level of antagonism over the control (without FA). In studies of the effect of the reduction in FA (400 ppm) level on chitinase gene expression in PCR assays, *nag1* was significantly repressed but *ech42* expression was only slightly repressed. Chitinase activity was either reduced or absent in the extracellular proteins of MSB supplemented with 400 ppm FA, which could be attributed to the effect of residual FA or its breakdown products through unknown mechanisms. Selection of S17TH as a toxin insensitive isolate that could commensurate the negative effect on chitinase activity makes it a potential antagonist against *Fusarium* spp.

Keywords Fusaric acid · Reduction · *Trichoderma harzianum* · Chitinase

Introduction

Phytotoxins are toxic compounds produced by fungi for self-defense by suppressing induced resistance or for plant pathogenesis by dissolving cell membranes. Fusaric acid (FA; 5-butyl-picolinic acid; C₁₀H₁₃NO₂, Mr: 179.22) is a well-known phytotoxin produced by several *Fusarium* spp., particularly pathogenic strains of *Fusarium oxysporum*, the causal agent of wilt diseases in a wide variety of plants [1]. In addition to its toxicity to plants, FA is toxic to microorganisms by inhibiting their growth and metabolism [2]. In biocontrol agents, such toxicity also represses the production of antifungal metabolites [3]. Detoxification or inactivation of phytotoxins is one of the mechanisms by which microbes protect themselves from the toxins [4] and therefore serves as an attractive strategy for decontamination of agricultural commodities and protection of crops from phytotoxic effect of fungal metabolites produced by pathogens.

Chitinases are cell wall degrading enzymes that degrade chitin polymers and are key components of mycoparasitism mechanism in select fungi and bacteria. *Trichoderma* species, which can act as potent biocontrol agents, exhibit mycoparasitism against a wide range of plant pathogens, mainly through their ability to produce chitinases [5, 6]. In addition to the production of these mycolytic enzymes, inactivation of pathogen toxins by *Trichoderma* spp. has also been reported [7] as one of the strategies for their biocontrol efficacy. However, no reports are available describing the relationship between the reduction in FA level and chitinase activity in any of the biocontrol agents, where studies on the effect of mycotoxins (phytotoxins) on chitinases are limited. Nonetheless, repression of *N*-acetyl- β -D-glucosaminidase (NAGase) in *Trichoderma atroviride* by trichothecene deoxynivalenol produced by *Fusarium* spp. was previously reported [3].

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In this paper, we investigated the ability of an antagonistic *T. harzianum* isolate S17TH in reducing the level of synthetic FA and assessed the role of this reduction on its chitinase activity.

Materials and Methods

Fungal Isolates

Fusarium oxysporum f. sp. *dianthi* (FOD) isolate CAFO-IHBT (MTCC 6659; GU048879) causing vascular wilt of carnation was used as the test pathogen. Five antagonistic *Trichoderma* isolates, S17TH, GITX-Panog (C), GITX-Panog (I), GITX-Kohli1, and GITX-Kohli 2 (Table 1) along with a non-antagonistic isolate GITX-Panog (J) were tested for FA inactivation.

Selection of *Trichoderma* Isolates for FA Tolerance

The isolates were tested in vitro for their ability to tolerate FA (*Gibberella fujikuroi*, Sigma). FA was added to solid minimal synthetic broth (MSB) (0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.9 g K_2HPO_4 , 0.2 g KCl, 1.0 g NH_4NO_3 , 2 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 2 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 2 mg $\text{MnCl}_2 \cdot 7\text{H}_2\text{O}$ supplemented with 0.1 % glucose; pH 5.6 g/l) at 50, 100, 250, 500, and 1,000 ppm. Fungal discs of 8 mm diameter were punched separately from 7-day old cultures and placed in the middle of the media plates. Medium without the toxin but inoculated with the individual isolate was maintained as a control. The inoculated plates were incubated at $28 \pm 2^\circ\text{C}$ and the linear mycelial growth was measured when the respective control plate showed full growth.

To determine mycelial weight, conidial suspension (1×10^5 conidia/ml) of each of the five isolates was inoculated in 100 ml of MSB with 500 ppm FA. MSB alone served as control. After incubating at $28 \pm 2^\circ\text{C}$ for 7 days

on a rotary shaker (180 rpm) in the dark, the mycelium was filtered through pre-weighed filter paper and the mycelial weight was recorded after drying at 70°C for 10 h.

TLC and HPLC Analyses of CFC Filtrate for Reduction in FA Level

Since the FA-tolerant *T. harzianum* isolate (S17TH) was unable to reduce 500 ppm FA added to MSB to a significant level, conidial suspension (1×10^5 conidia/ml) of the isolate was inoculated in 100 ml MSB containing 400 ppm of FA and incubated as previously described. Uninoculated MSB containing 400 ppm FA served as a control. Cell-free culture (CFC) filtrate was prepared [8] and the toxin was isolated and characterized from this filtrate as previously described [9]. Commercial FA (Sigma) was used as a standard. High-performance liquid chromatography (HPLC) analysis was performed on a Shimadzu Prominence HPLC system. All samples and standards were filtered through $0.45 \mu\text{m}$ Millipore (Bangalore, India) filters. The separation was achieved on Zorbax Extend C-18 column ($250 \text{ mm} \times 4.6 \text{ mm}$; $5 \mu\text{m}$ particle size) from Agilent (Palo Alto). The mobile phase used for analysis was 35:75 v/v acetonitrile:water (1 % K_2HPO_4 ; pH adjusted to 7.35 with concentrated phosphoric acid) by isocratic elution at a flow rate of 0.7 ml/min. The detection (UV-detector) of analyte was performed at 275 nm. FA content in the sample was quantified using a linear calibration curve prepared with FA concentrations ranging from 2 to 5 μg .

Bioassays of CFC Filtrate for Toxicity

The filtrate with reduced FA levels was tested on callus tissues of carnations. Callus initiation was carried out as described [10]. Calluses were initiated after 10 days of inoculation but profuse growth was observed after 35 days. FA (400 ppm) and the S17TH treated filtrate were tested on callus tissues for survival [10] and electrolyte leakage [11].

Table 1 Selection of *Trichoderma* isolates for FA tolerance

Isolate	Identity	Mycelial growth (mm) on FA (ppm) ^a				Mycelial weight (mg) ^a /100 ml	
		50	100	250	500	MSB+FA (500 ppm)	MSB (control)
S17TH (MTCC 7679)	<i>Trichoderma harzianum</i>	48 ± 2	47 ± 1	46 ± 2	38 ± 1	1.6 ± 0.2	1.7 ± 0.1
GITX-Panog (C) (MTCC 8616)	<i>Trichoderma saturnisporum</i>	55 ± 2	55 ± 2	45 ± 1	25 ± 2	0.6 ± 0.2	1.5 ± 0.2
GITX-Panog (I) (MTCC 8611)	<i>Trichoderma longibrachaeum</i>	55 ± 1	48 ± 1	37 ± 1	8 ± 1	0.0 ± 0	1.6 ± 0.1
GITX-Kohli 1 (MTCC 8612)	<i>Hypocrea jecorina</i>	55 ± 1	55 ± 2	45 ± 1	27 ± 2	1.1 ± 0.2	1.6 ± 0.2
GITX-Kohli 2 (MTCC 8613)	<i>Hypocrea jecorina</i>	55 ± 1	55 ± 1	45 ± 2	24 ± 2	0.6 ± 0.2	1.5 ± 0.2
LSD ($P = 0.05$)					1.8	0.1	0.2

^a Mean of three replicates; GITX-Panog (J) not included for analyses; MTCC microbial type culture collection, Institute of Microbial Technology, Chandigarh, India

MSB alone served as a control. Electrical conductivity was measured after 30 min of incubation using conductivity meter (Cyberscan 510) and the electrolyte leakage was expressed as $\mu\text{Siemens } 100 \text{ mg}^{-1}$ of callus tissue.

Chitinase Gene Expression in Relation to Reduced FA Level

Total RNA was isolated from mycelial samples (100 mg) of S17TH grown in MSB containing 1 % colloidal chitin, supplemented with 100–400 ppm FA by Tri-reagent (Invitrogen) according to the manufacturer's protocol. MSB with 1 % colloidal chitin served as a control. For semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR), 2 μg of total RNA was added to oligo (dT) 12–18 primer and the sample was briefly denatured at 65 °C for 10 min and chilled on ice for 2 min. Reverse transcription was carried out with MuMLv (Fermentas) at 42 °C for 1 h. Two μl of an eightfold dilution of this cDNA was used as the template for amplification by PCR in a 25 μl reaction with ready-mix (Qiagen). Analysis of the transcription of the *Trichoderma* actin-encoding gene was performed by amplification using primers, TVactS 5'-GTCAGGTCATCACCATCGGCAACG-3' and TVactAS 5'-GCTGGAAGGTGGACAGGGAGGC-3' and used to normalize the amount of cDNA added to reactions. Endochitinase (*ech42*) was amplified using primers endochiF (5'-AAGATTGTTCTCGGCATGCCCATC-3') and endochiR (5'-GGGTATCGAAAGAGATGAGCTCCTT-3') at 94 °C for 4 min, 32 cycles of 94 °C for 15 s, 62 °C for 40 s, 72 °C for 1 min, with a final extension at 72 °C for 5 min. Exochitinase (*nag1*) was amplified with primers, exochiF (5'-ATGCTGCCCAAGGCGATCATC-3') and exochiR (5'-TAAAATAGTGTGCAGGCAGTG-3'), at 94 °C for 4 min, 32 cycles of 94 °C for 15 s, 52 °C for 40 s, 72 °C for 2 min, with a final extension at 72 °C for 5 min.

Quantitative RT-PCR assay for gene expression was performed in a BioRad iCycler with the following programme: initial denaturation for 180 s at 95 °C, 50 cycles of 95 °C for 20 s, 60 °C for 20 s and 72 °C for 20 s. Amplification was carried out with the primer pairs, sense-5'-CGCAACTTCAGCCTCAGAACC-3' and antisense-5'-TCAATACCATCGAAACCCAGTCC for *ech42*, and with the sense-5'-TGTCCTACAGCCTCTGCTGCAAAAGTTC and antisense-5'-CATCTCCTCACAGACAAGCGGTGAAAG primers for *nag1*. The 20 μl of reaction volume included 2 μl of cDNA, 10 μl of 2XABsolute SYBR Green ROX MIX (Thermo) and 100 nM final concentration of specific primers for the gene of interest. Actin amplified with the primers sense 5'-GCACGGAATCGCTCGTTG and antisense 5'-TTCTCCACCCCGC-CAAGC was used as a house-keeping gene to normalize the amounts of cDNA. The reactions were performed in triplicate and transcript abundance was calculated as described [12].

Chitinase Production and Detection in Relation to Reduction in FA Level

Chitinase production by S17TH was quantitatively evaluated in the extracellular proteins of the cell free culture filtrate of the above experiment. Chitinase activity was detected by a chitinase assay kit (Sigma) following the manufacturer's protocol. Protein separation was carried out by 7.5 % SDS-PAGE co-polymerized with 100 μM of either of the fluorogenic substrates (Sigma): 4-methylumbelliferyl-*N*-acetyl- β -D-glucosaminide 4-MU-(GlcNAc) and 4-methylumbelliferyl- β -D-*N,N',N''*-triacylchitotriose [4-MU-(GlcNAc)₃] for NAGase and chitotriosidase, respectively. Chitinase activity was reactivated by removing SDS upon incubating the gel with 1 % Triton-X in 100 mM Tris buffer, pH 8.0, as previously described [13] followed by incubation at 37 °C for 30 min in 100 mM sodium acetate buffer (pH 5.0). The enzyme activity appeared as a fluorescent band under UV light because of the enzymatic hydrolysis of 4-methyl umbelliferone from the GlcNAc disaccharide.

Antagonistic Efficacy of S17TH

The isolate selected for analysis of the reduction in FA level was also evaluated for its antifungal activity against FOD in comparison to a non-tolerant (GITX-Panog-J) isolate by dual culture assays on potato dextrose agar (PDA) [8] in the presence (400 ppm) or absence of FA. The time of inoculation of the pathogen in dual culture was decided based on its growth rate with respect to the antagonist in monoculture. Plates inoculated with the pathogen alone served as controls. The level of antagonism displayed by the *Trichoderma* spp. was recorded as previously described [8], when the control plate showed full growth.

Statistical Analyses

All experiments were repeated at least twice at different time periods in triplicate. Data were analyzed by one-way analysis of variance (ANOVA). Differences between treatment mean values were determined following least significant difference LSD test at $P < 0.05$.

Results

Selection of *Trichoderma* Isolates for FA Tolerance

In general, all the antagonistic isolates were able to tolerate FA up to a maximum concentration of 500 ppm. However, a *T. harzianum* isolate S17TH recording less than 50 % reduction in mycelial growth and weight in FA amended

solid and liquid MSB, respectively, over control (MSB alone) was considered to be FA tolerant (Table 1). On solid MSB, a consistent mycelial growth of S17TH was also observed up to 250 ppm concentration of FA. None of the isolates could tolerate 1,000 ppm of FA. Interestingly, complete inhibition of mycelial growth was noticed for the non-antagonistic isolate (GITX-Panog J) at all tested concentrations of FA (data not shown). The reduction in FA content was assessed by TLC and HPLC analyses and by bioassays involving carnation callus survival and electrolyte leakage as indicators.

TLC and HPLC Analyses for Reduction in FA Level

The antagonistic isolate S17TH, despite the ability to tolerate 500 ppm FA, did not induce significant changes in the toxin as observed by TLC and HPLC analyses. However, when the concentration of FA in the broth was reduced to 100–400 ppm, the isolate showing a mycelial weight of 3.9–2.1 mg could reduce the level of FA in the filtrate within 7 days by unknown mechanisms as observed by TLC and HPLC analyses. TLC analyses for various solvents of the filtrate from MSB amended with 100–300 ppm did not detect FA in the *n*-butane and ethyl acetate fractions (data not shown). Though FA was detected in the *n*-butane and ethyl acetate fractions of the filtrate from MSB with 400 ppm FA (Fig. 1a), the spot size of FA was significantly smaller in these samples than the one observed for commercial FA or MSB+FA (control). This was corroborated by HPLC analyses, where the FA level was determined to be only 12.1 % in the *n*-butanol extract, 11.5 % was detected in the ethyl acetate fraction (Fig. 1b) in comparison to control thereby ascertaining the role of S17TH in reducing the FA content. No extraction of FA was observed in *n*-hexane.

Bioassays for the Reduction in FA Level

In studies on callus tissues, the survival was found to be maximum (76.6 %) in the S17TH treated filtrate in comparison to FA (23.3 %). Untreated callus (control) recorded maximum survival (90 %). These results were further corroborated by the levels of ion (electrolyte) leakage measured as an increase in conductance after challenged with FA and the S17TH treated filtrate. While the toxin treated sample showed maximum loss of electrolytes (247.6 μ S), the leakage was observed to be drastically reduced for the S17TH treated filtrate (98.3 μ S) and control (37.4 μ S) cultures (Table 2). On treating the macerated callus tissues incubated in MSB alone, MSB+FA, and S17TH treated filtrate with 0.1 % erythrocine B, the cells derived from callus tissues incubated in MSB+FA did stain after 48 h, whereas no staining was observed in the cells incubated in MSB alone and S17TH treated filtrate

(Fig. 2). Non-staining of cells incubated in S17TH treated filtrate further confirmed the non-toxicity of the filtrate to carnation cells.

Chitinase Production and Detection in Response to Reduction in FA Level

Semi-quantitative RT-PCR assays for the expression of the endochitinase, *ech42* (234 bp) showed that the enzyme was slightly repressed at 400 ppm FA. Expression of the exochitinase, *nag1* (1,735 bp) was completely repressed at 300 and 400 ppm FA (Fig. 3a). In quantitative RT-PCR assays, though no downregulation of *nag1* could be observed at 400 ppm FA, a significant reduction in transcriptional induction over the basal level suggested the repressive effect of the phytotoxin (Fig. 3b). Repression of chitinase genes after 7 days of culture, i.e., the minimal period required for reduction in FA level (400 ppm), could be attributed to the residual toxin detected in the filtrate by HPLC analysis or the breakdown products of FA. In chitinase assays for FA supplemented MSB, the production of the exochitinase, NAGase was significantly reduced (19.5-fold) in comparison to control cultures. No significant reduction in chitobiosidase (exochitinase) and chitotriosidase (endochitinase) production over the control was noticed for FA amended MSB. Similar levels of chitobiosidase and chitotriosidase production (0.1 U/ml of filtrate) were observed in the treatment compared with their respective controls, with 0.5 and 0.2 U/ml filtrate, respectively (Table 3). No chitinases were detected in zymogram analyses of extracellular proteins precipitated from the S17TH treated filtrate (400 ppm), however, two chitinases of 73 and 42 kDa were detected in untreated controls (Fig. 4). Since reduction in chitinase activity in presence of toxin occurs at RNA level, the possibility of less mycelial growth attributing to reduced chitinase activity is negated.

Antifungal Efficacy of S17TH

In dual culture assays for antagonism of S17TH against FOD on PDA supplemented with 400 ppm FA, the isolate showed class I level of antagonism, i.e., the antagonist grew similar to controls grown in the absence of FA. This indicated that its antifungal efficiency was less influenced by the presence of FA (Fig. 5) in the medium.

Discussion

The phytotoxic FA is so effective that it is used as a selective agent to obtain plants resistant to *Fusarium* spp. The *in planta* production of FA by several *formae speciales* of *F. oxysporum* has been reported [14]. Screening for

Fig. 1 Analyses of FA. **A** TLC for fusaric acid detection in S17TH treated filtrate. *Lanes: S* commercial fusaric acid/MSB+FA (untreated sample); *1*n-hexane extraction; *2* ethyl acetate extraction; *3*n-butanol

extraction; *4* water fraction. **B** HPLC analyses of FA. **a** commercial fusaric acid (Sigma); **b** crude sample; **c** ethyl acetate fraction; **d**n-butanol fraction

Table 2 Fusaric acid toxicity to carnation tissues

Treatment	Callus survival (%) ^a	Electrolyte leakage (μS) ^a after 30 min
MSB+FA (400 ppm) + S17TH	76.6 ± 2.2	98.3 ± 2.8
MSB+FA (400 ppm)	23.3 ± 2.1	247.6 ± 2.6
MSB (control)	90.0 ± 2.4	37.4 ± 1.7
LSD (<i>P</i> = 0.05)	2.0	2.0

^a Mean of three replicates

Fig. 2 Erythrosine staining of 0.5 % macerozyme-treated carnation callus. **a** MSB only (negative control); **b** FA treated; **c** S17TH treated filtrate

toxin-insensitive microbes is one of the strategies for increased reliability of antagonists or biocontrol agents targeting *Fusarium* diseases. These prompted us to screen the antagonistic *Trichoderma* spp. effective against the pathogen for inactivation of its metabolite at 400 ppm, a higher concentration than is phytotoxic [1]. In this study, the significant reduction in FA content incited by a *T. harzianum* isolate S17TH was corroborated by reduced severity of toxin-induced effects in the bioassays.

Resistance to toxins can be due to various physiological reactions such as lack of uptake, insensitivity of the target and detoxification [15]. The reduction in FA content coupled with reduced electrolyte leakage observed in this study indicates the possibility of detoxification. Microbial detoxification of phytotoxins produced by the pathogenic microbes has been reported [4, 7, 16].

Selection of antagonistic *Trichoderma* detoxifying FA with no suppression in antifungal gene expression would be

Fig. 3 PCR assays for the expression of antifungal chitinases in S17TH. **a** Semi-quantitative. Lanes: 1 control (MSB alone); 2 100 ppm; 3 200 ppm; 4 300 ppm; 5 400 ppm. **b** Quantitative RT-PCR assays for induction of the enzymes by FA (400 ppm). The y-axis indicates the transcript levels normalized by actin; the error bars are SD for three replications

Table 3 Chitinase production by FA reducing S17TH

Treatment	Enzyme activity (U mL ⁻¹ of culture filtrate) ^b		
	<i>N</i> -acetyl glucosaminidase	Chitobiosidase	Chitotriosidase (endochitinase)
MSB+FA (400 ppm) ^a	0.2 ± 0.1	0.1 ± 0	0.1 ± 0.1
MSB ^a (positive control)	3.9 ± 0.1	0.5 ± 0.1	0.2 ± 0.1
MSB alone (negative control)	0.0 ± 0	0.0 ± 0	0.0 ± 0
LSD (<i>P</i> = 0.05)	0.1	0.1	0.2

^a Contained 1 % colloidal chitin

^b Mean of three replicates; one unit of the enzyme was defined as the amount of enzyme that will release 1.0 μmol of *p*-nitrophenol from the appropriate substrate per min at pH 5.0 at 37 °C

an added advantage. However, the S17TH isolate despite showing FA tolerance also displayed partial reduction in mycelial growth in FA amended solid and liquid MSB over control (MSB alone). Toxic metabolites that influence the mycelial growth have a considerable influence on enzymatic activity. Repression of antifungal metabolite production due

Fig. 4 Zymograms of extracellular protein of *Trichoderma harzianum* S17TH. Whole protein (0.1 μg) was detected for **a** *N*-acetyl glucosaminidase and **b** endochitinase with the substrates 4-MU-(GlcNAc) and [4-MU-(GlcNAc)₃], respectively. Lanes: M native protein marker (Genei); 1 untreated control; 2 S17TH grown on MSB containing 400 ppm of FA

to toxicity has been reported in biocontrol agents [3]. Since expression of the chitinase genes, *chi42* [17] and *nag1* [18], have been demonstrated to contribute to disease suppression in *Trichoderma*, their expression during reduction in FA level was analyzed using the RNA samples. While *ech42* expression was slightly repressed at 400 ppm of FA, *nag1* was significantly repressed at 300 ppm. In chitinase assays, significant reduction was observed only for NAGase production. Further native gel analyses of the extracellular proteins from untreated control detected two chitinases of 73 (NAGase) and 42 kDa (endochitinase), which were absent in the S17TH treated filtrate. Proteins with molecular mass of 73 and 42 kDa were previously reported to be NAGase and endochitinase, respectively [5]. In *Trichoderma* spp., since chitinases are known to be induced by stress [19], the repression of chitinases therefore excludes the possibility of stress and could be elicited by the residual FA or its breakdown products through unknown mechanisms. Earlier, trichothecene deoxynivalenol, a yet another toxic metabolite of *Fusarium* spp. was reported to repress *nag1*, but did not affect the expression of *ech42* in *Trichoderma atroviride* P1 [3].

Since chitinases play significant role in the mycoparasitism of *Trichoderma* spp. and in view of the repression of its production due to reduction in FA level, the S17TH was assayed for its antagonistic activity. Interestingly, in dual culture studies, the isolate displayed a similar level of class I antagonism against the pathogen in the presence or absence of FA. This observation could be attributed to its tolerance to FA that compensates for the repressive effect of the toxin on chitinases. The inactivation of pathogen toxins combined with biocontrol efficacy has earlier been established [20].

Fig. 5 Antagonism of *Trichoderma* spp. against *Fusarium oxysporum* f. sp. *dianthi* (CAFO-IHBT) in dual culture. S17TH exhibit class I level of antagonism i.e., covering the media surface and the non-antagonistic one (GITX-Panog J) exhibit class 3 level of antagonism. i.e., *Trichoderma* and *Fusarium* sp. colonized each one half of the media surface and did not have dominance. Classification was followed as described earlier (Shanmugam et al. 2008). A Pathogenic control (CAFO-IHBT); B S17TH (antagonistic) vs. CAFO-IHBT in absence of fusaric acid; C S17TH (antagonistic) vs. CAFO-IHBT in presence of fusaric acid; arrows indicate sporulation; D GITX-Panog (J) (non-antagonistic) vs. CAFO-IHBT in absence of fusaric acid displaying class I level of antagonism; E GITX-Panog (J) vs. CAFO-IHBT in presence of fusaric acid showing no growth of the non-antagonistic isolate

The effect of synthetic FA on chitinase expression observed in the current study is likely to encourage further studies on molecular interactions between FA producing pathogens and the fungal antagonist. Chitinase expression in relation to the reduction in FA level targeting different strains and studies on changes in gene expression at the level of transcription using a reporter system are the other areas, which need to be elucidated. Selection of S17TH as a toxin insensitive isolate that could commensurate the negative effect on chitinase activity makes it a potential antagonist against *Fusarium* spp.

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References

1. Fakhouri W, Walker F, Armbruster W, Buchenauer H (2003) Detoxification of fusaric acid by a nonpathogenic *Colletotrichum* sp. *Physiol Mol Plant Pathol* 63:263–269
2. Bacon CW, Hinton DM, Hinton AJr (2006) Growth-inhibiting effects of concentrations of fusaric acid on the growth of *Bacillus mojavensis* and other biocontrol *Bacillus* species. *J Appl Microbiol* 100:185–194
3. Lutz MP, Feichtinger G, Defago G, Duffy B (2003) Mycotoxinogenic *Fusarium* and deoxynivalenol production repress chitinase

- gene expression in the biocontrol agent *Trichoderma atroviride* P1. *Appl Environ Microbiol* 69:3077–3084
4. Aggarwal R, Gupta S, Singh VB, Sharma S (2011) Microbial detoxification of pathotoxin produced by spot blotch pathogen *Bipolaris sorokiniana* infecting wheat. *J Plant Biochem Biotechnol* 20(1):66–73
5. Seidl V (2008) Chitinases of filamentous fungi: a large group of diverse proteins with multiple physiological functions. *Fungal Biol Rev* 22:36–42
6. Sharma V, Shanmugam V (2012) Purification and characterization of a 24 kDa chitobiosidase from the mycoparasitic fungus *Trichoderma saturnisporum*. *J Basic Microbiol* 52:324–331
7. El-Hasan A, Walker F, Buchenauer H (2008) *Trichoderma harzianum* and its metabolite 6-phenyl- α -pyrone suppress fusaric acid produced by *Fusarium moniliforme*. *J Phytopathol* 156:79–87
8. Shanmugam V, Sharma V, Ananthapadmanaban (2008) Genetic relatedness of *Trichoderma* isolates antagonistic against *Fusarium oxysporum* f.sp. *dianthi* inflicting carnation wilt. *Folia Microbiol* 53(2):130–138
9. Amalfitano C, Raffaele P, Anna A, Maurizio V, Maria CZ, Antonio E (2002) HPLC analysis of fusaric acid, 9,10-dehydro-fusaric acid and their methyl esters, toxic metabolites from weed pathogenic *Fusarium* species. *Phytochem Anal* 13:277–282
10. Thakur M, Sharma DR, Sharma SK (2002) In vitro selection and regeneration of carnation (*Dianthus caryophyllus* L.) plants resistant to culture filtrate of *Fusarium oxysporum* f.sp. *dianthi*. *Plant Cell Rep* 20:825–828
11. Shanmugam V, Raguchander T, Balasubramanian P, Samiyappan R (2001) Inactivation of *Rhizoctonia solani* toxin by a putative α -glucosidase from coconut leaves for control of sheath blight disease in rice. *World J Microbiol Biotechnol* 17:545–552
12. Shanmugam V, Ronen M, Shalaby S, Larkov O, Rachamlin Y, Hadar R, Rose MS, Carmell S, Horwitz BA, Lev S (2010) The fungal pathogen *Cochliobolus heterostrophus* responds to maize phenolics: novel small molecule signals in a plant-fungal interaction. *Cellular Microbiol* 12(10):1421–1434
13. Singh NA, Shanmugam V (2012) Cloning and characterization of a bifunctional glycosyl hydrolase from an antagonistic *Pseudomonas putida* strain P3(4). *J Basic Microbiol* 52:340–349
14. Amusa NA (2006) Microbially produced phytotoxins and plant disease management. *Afr J Biotechnol* 5:405–414
15. Samiyappan R, Balasubramanian P, Babu S, Nandakumar R, Shanmugam V, Raguchander T, Ramanathan A (2002) Advances in molecular biology of phytotoxin production and its involvement in plant pathogenesis. In: Upadhyay RK (ed) *Advances in microbial toxin research and its biotechnological exploitation*. Kluwer Academic/Plenum Publishers, New York, pp 231–258
16. Mohamed ZA, Alamri SA (2012) Biodegradation of cylindrospermopsin toxin by microcystin-degrading bacteria isolated from cyanobacterial blooms. *Toxicon*. doi:10.1016/j.toxicon.2012.10.004
17. Romao-Dumaresq AS, de Araujo WL, Talbot NJ, Thornton CR (2012) RNA interference of endochitinases in the sugarcane endophyte *Trichoderma virens* 223 reduces its fitness as a biocontrol agent of pineapple disease. *PLoS One* 7:e47888
18. López-Mondéjar R, Catalano V, Kubicek CP, Seidl V (2009) The β -N-acetylglucosaminidases *nag1* and *nag2* are essential for growth of *Trichoderma atroviride* on chitin. *FEBS J* 276:5137–5148
19. Gruber S, Kubicek CP, Seidl-Seiboth V (2011) Differential regulation of orthologous chitinase genes in mycoparasitic *Trichoderma* species. *Appl Environ Microbiol* 77(20):7217–7226
20. Matarese F, Sarrocco S, Gruber S, Seidl-Seiboth V, Vannacci G (2012) Biocontrol of *Fusarium* head blight: interactions between *Trichoderma* and mycotoxinogenic *Fusarium*. *Microbiology* 158:98–106