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Microbial detoxification of *Colletotrichum falcatum* toxin

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A study was conducted on the possible detoxification of phytotoxin produced by the sugarcane red-rot pathogen *Colletotrichum falcatum* Went by antagonistic fungal and bacterial strains. Eleven *Pseudomonas fluorescens* strains and two *Trichoderma harzianum* strains were grown on a medium containing the pathogen toxin. Later, the treated toxin was tested for its phytotoxic activity using symptom bioassay, electrolytic leakage and spectral analysis. In symptom bioassay, the phytotoxin incubated with *P. fluorescens* strains FP 7 and VPT 4 and *T. harzianum* strain T-5 did not exhibit any symptom on susceptible sugarcane leaves. These treatments caused reduction in electrolyte leakage compared to other treatments and untreated toxin. Also, spectral analysis showed varied spectral patterns by different treatments. The results revealed that certain strains of the biocontrol agents cause detoxification of the pathogen toxin, which is one of the major pathogenicity determinants of the red-rot pathogen.

DETOXIFICATION or inactivation of the phytotoxin reduces the toxicity of metabolite produced by plant pathogens. This process leads to development of resistant reaction or acts as a defence mechanism in susceptible plants to protect them from pathogen infection. Toxin resistance genes can be isolated from the pathogen itself, as well as from other microbes. Microorganisms form an exotic source of enzymes which are capable of inactivating synthetic chemicals that are potentially phytotoxic[1–3]. Studies are now being carried out on transformation of plants utilizing phytotoxin resistance genes of microbial origin.

The red-rot pathogen *Colletotrichum falcatum* Went is known to produce a phytotoxic metabolite identified as an anthroquinone compound[4]. It has been established that the toxic metabolite is host-specific and produces part of the disease symptoms[5]. Both fungal and bacterial antagonists are being studied for the possible protection of sugarcane against the red-rot pathogen. Recently, strains of *Pseudomonas* spp. and *Trichoderma harzianum* effective against the pathogen have been identified[6,7]. In this context, further studies were made on the possible inactivation of the pathogen toxin by these antagonistic strains.

The antagonistic strains of biocontrol agents, including two isolates of *T. harzianum* (T-5 and T-62) and 11 isolates of *Pseudomonas fluorescens* (ARR 1G, ARR 2, ARR 10, CHAO, EP 1, FP 7, KKM 2, Pfl, VPT 2, VPT 4 and VPT 10) isolated from sugarcane rhizosphere were employed for this purpose[6,7]. Regarding the phytotoxin, the partially purified toxin from the *C. falcatum* pathotype Cf 671 was obtained and utilized in this study[5,8].

For growing the antagonists in the toxin-amended medium, the fluorescent pseudomonads and *T. harzianum* isolates were inoculated in synthetic medium (K_2HPO_4, 1.50 g; $MgSO_4$, 1.50 g; $NaNO_3$, 2.00 g; $FeSO_4$, 0.01 g; distilled water, 1000 ml incorporated with the toxin @1000 ppm) and incubated for eight days at room temperature. All the isolates were inoculated at equal concentration in 100 ml of medium and incubated as stationary cultures. The cultures were centrifuged, supernatants filter-sterilized and used for bioassay, absorption spectral analysis and for the estimation of electrolyte leakage (EC). The pellets were used for the estimation of cell concentration.

After centrifugation, pellets of all the *P. fluorescens* isolates were suspended in a known quantity of distilled water and cell concentration was estimated by examining the turbidity at 595 nm with a spectrophotometer[9]. Growth of *T. harzianum* isolates was observed visually.

Bioassay was carried out on sugarcane leaves as symptom production using antagonists treated and untreated *C. falcatum* toxin. The highly susceptible sugarcane cultivars, viz. CoC 86062 and CoC 92061 were used for this study. The youngest, fully expanded leaves were chosen and four leaf segments from four different plants were used for each treatment. Twenty µl of phytotoxin solution was placed over each spot on segments of the sugarcane leaves injured with fine pinpricks and incubated at

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30°C in moist chambers[5]. The symptom development was observed within 48–72 h.

Loss of electrolytes induced by toxin was assessed by measuring the conductivity (micro Siemens) of the medium into which electrolytes were released from the cut bits[10] in which random samples with leaf discs of 4 to 5 mm diameter were cut, rinsed and placed in scintillation vials. One hundred mg of leaf-disc tissue contained in each vial was vacuum infiltrated with 5 ml of toxin solution at 1000 ppm concentration or water for 30 min. The toxin solution or water was removed, discs were rinsed for 10 min with several changes of distilled water and 5 ml of water was added as leaching solution. Vials were incubated on a reciprocating shaker (120 strokes/min) for 30 min. After 30 min, changes in conductance values of the leachate were recorded at 10, 20 and 30 min time intervals.

To study changes in the toxic metabolite, the treated and untreated toxin samples were subjected to spectral analysis in a SpectraMAX 190 Microplate Reader (Molecular Devices, USA). Spectral pattern and absorption maxima were recorded between 190 and 350 mm.

Results of the present study revealed that all the bacterial isolates were able to grow in the toxin medium and the growth rate varied among the strains (Table 1). The strains CHAO and VPT 2 showed maximum growth in the medium. Growth of fungal and bacterial antagonists in toxin medium showed that the antagonists have tolerance to the *C. falcatum* toxin. It has been reported that a strain of *Bacillus subtilis*, BG 3 was able to survive on a brefeldin A (BFA acid) medium by simply converting it to a non-toxic metabolite by means of a brefeldin A esterase (bfs esterase), which is a single-step hydrolysis reaction[11]. BFA is a non-specific phytotoxin from *Alternaria carthami* causing leaf and stem blight of safflower. While screening diverse soil microorganisms for fusaric acid (FA) resistance, it was observed that *Cladosporium wernickii* and *Klebsiella oxytoca* survived using FA as the sole source of carbon[12]. Sriram *et al.*[13] reported that a few isolates of *Trichoderma viride* were found to inactivate the *Rhizoctonia solani* toxin which formed the sole food source of the biocontrol fungus.

Spot application of untreated toxin on leaf segments produced brown, necrotic spots with a yellowish-brown margin and a yellow halo, which elongated along the veins within 48–72 h. The antagonistic strain-treated toxin caused a general reduction in symptom production in both the varieties. However, a clear variation in residual toxin activity was recorded (Table 2). Phytotoxin samples treated with *P. fluorescens* strains FP 7 and VPT 4 and *T. harzianum* strain T5 did not produce any symptom on the leaves. However, the toxin samples treated with strains Pf1, ARR 1G, ARR 2 and T62 could not reduce symptoms in CoC 86062 and in CoC 92061. The remaining strains were moderate in their action. Symptom bioassay for this phytotoxin has been used by various workers[4,5,14] and they have established that part of the disease symptoms on the host could be produced by using the partially purified toxin. It suggests that the reduction of symptoms may be possibly due to inactivation of the toxic metabolite.

Electrolyte leakage studies revealed that there was an overall reduction in loss of electrolytes caused by the growth of antagonists in toxin medium (Figure 1). Here also, the strains FP 7, VPT 4 and T5 were efficient in reducing loss of electrolytes and strains ARR 1G, Pf1 and VPT 2 had shown similar results. Conversely, the strains CHAO, EP 1 and T62 showed increased loss in

Table 1. Growth of bacterial strains in toxin-incorporated medium

P. fluorescens isolate	Cell concentration ($\times 10^8$ cfu/ml)
ARR 1G	1.3
ARR 2	1.1
ARR 10	2.4
CHAO	2.9
EP 1	1.5
FP 7	1.1
KKM 2	1.2
Pf1	1.9
VPT 2	3.3
VPT 4	1.6
VPT 10	2.5

Normal growth of bacterial isolates in King's B broth ranges from 2 to 3 $\times 10^8$ cfu/ml.

Table 2. Symptom bioassay with detoxified toxin by antagonists

Antagonist	Symptom development	
	CoC 86062	CoC 92061
P. fluorescens		
ARR 1G	+++	+
ARR 2	+++	+
ARR 10	++	–
CHAO	+	+
EP 1	+++	–
FP 7	–	–
KKM 2	+	+
Pf1	+++	–
VPT 2	+	+
VPT 4	–	–
VPT 10	++	+
T. harzianum		
T5	–	–
T62	+++	–
Toxin alone	+++	+++

+++, Elongated, brown, necrotic spots with yellow halo; ++, Small, brown spots; +, Yellowing without necrosis; –, No symptom.

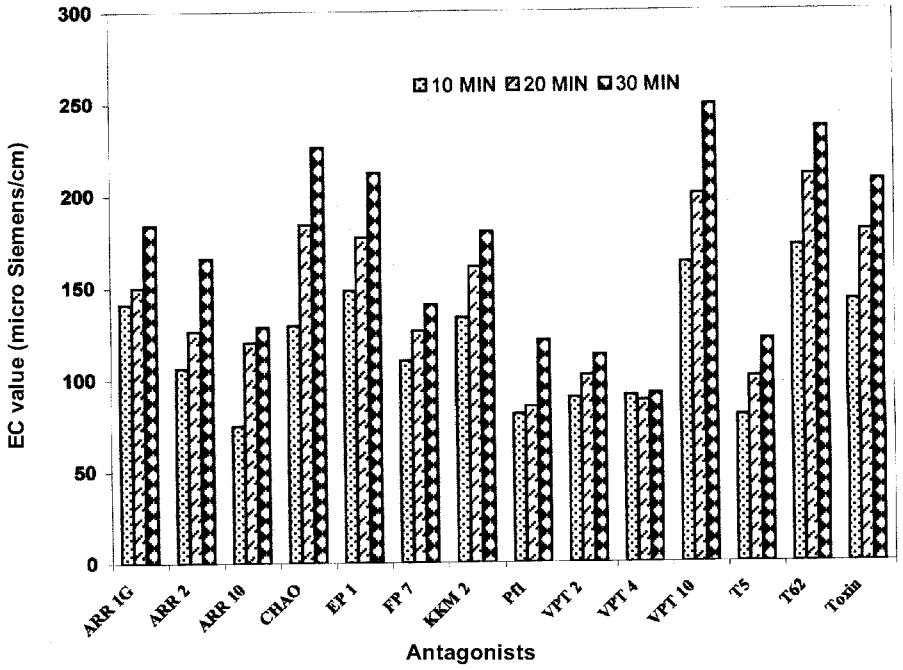

Figure 1. Effect of antagonists on toxin-induced loss of electrolytes.

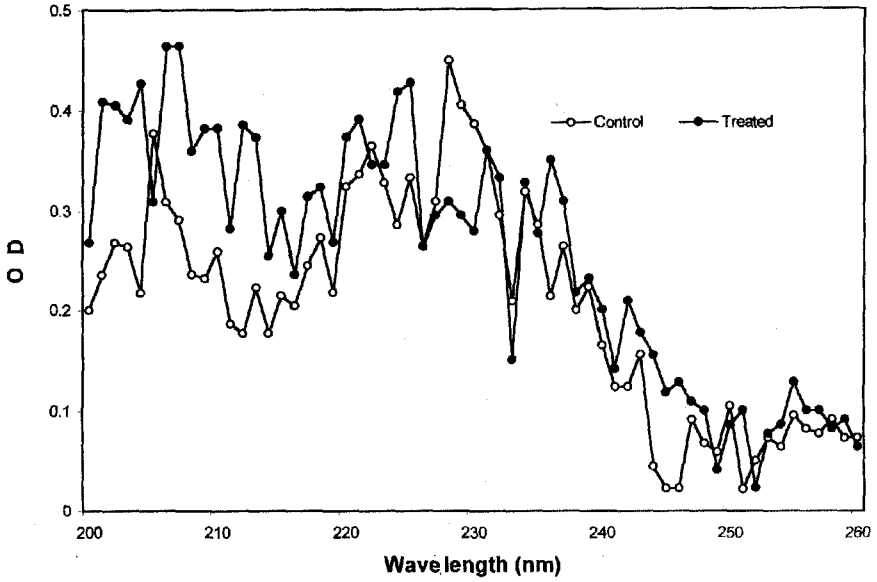

Figure 2. Spectral pattern of untreated and biocontrol agent (bca)-treated toxin.

electrolytes over control. In general, there was an overall increase in leakage of electrolytes with increase in time interval/duration. Progressive increase in the loss of electrolytes due to pathogen toxin was reported in calluses and leaves of sugarcane[14] and rice[15]. In the present investigation, results on the reduction of both symptom production and electrolytic leakage by the antagonistic strains were positively related. A reduction in severity of toxin-induced symptoms by the biocontrol agents and their efficacy in bringing about the sharp fall in electrolytic leakage was reported with RS toxin produced by *R. solani*, the rice sheath blight pathogen[13]. The positive correlation between symptom production and loss of electrolytes had been reported in various crops such as oats by Victorin[16]; tobacco, cucumber and *Solanum* sp. by *Colletotrichin*[17]; sorghum by PC toxin[18] and tomato by fusaric acid[19].

The growth of antagonists in the toxin medium caused a shift in spectral pattern (Figure 2) and absorption maxima (Figure 3) when compared to toxin-free medium.

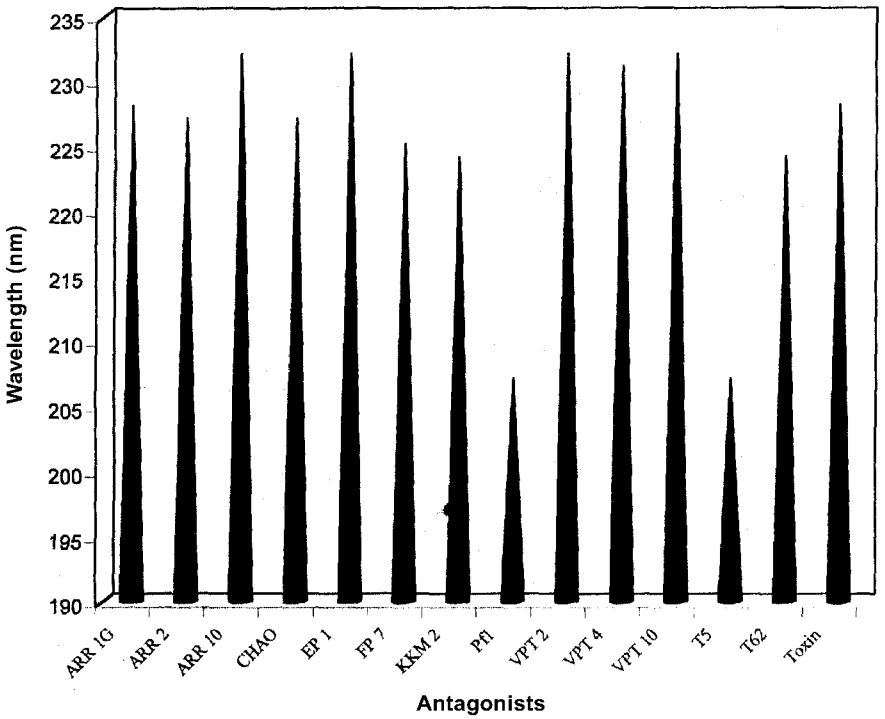

Figure 3. Absorption maxima of toxin treated with antagonistic microbes.

However, the change was not uniform with different treatments. Absorption spectral analysis showed that the peaks and absorption maxima of all the treated toxins varied from each other and no similarity was obtained with the untreated toxin. Spectrophotometric analysis of toxin with similar peaks has been reported by earlier workers[4,14]. Results of the present study also confirm this. Hence the variation in absorption maxima and peaks of toxin grown with the antagonists are attributed to changes in the chemical nature of the toxin, which result in alteration in infection process (or) its activity in relation to symptom production.

The biocontrol efficacy of the microbial antagonists selected has already been studied and their antagonistic activity against *C. falcatum* proved *in vitro*[20]. Under pot-culture conditions also their efficacy has been established against red-rot disease of sugarcane. The detoxification of pathogen toxin combined with biocontrol efficacy has been well established with *Pantoea dispersa*, which offered an excellent biocontrol against sugarcane leaf scald disease caused by *Xanthomonas alblineans*. In this host–pathogen interaction, the antagonistic bacterium detoxified albicidin toxin produced by the pathogen[21]. Several bacterial strains capable of degrading oxalic acid associated with pathogenesis were able to protect *Arabidopsis thaliana* from infection caused by *Sclerotinia sclerotiorum*[22]. Few species of *Penicillium*, *Trichoderma* and *Ralstonia pickettii* were able to grow on thaxotamin A secreted by *Streptomyces scabies*, a potato pathogen. However, the bacterium was alone able to protect the plants from the scab[23]. It shows non-correlation in ability of the antagonists to degrade toxin and to control the disease, which should be clarified for the successful transgenic approach. This lack of correlation has been observed among some strains of antagonists tested in this study and their biocontrol efficacy.

Results of this study confirm the efficacy of some strains of biocontrol agents in detoxifying the pathogen toxin. In continuation, further studies are being carried out to characterize the active compounds in the antagonists responsible for detoxification of the toxic metabolite. This toxin inactivation principle gene may serve as a candidate gene in developing transgenic sugarcane for red-rot resistance.

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Genotypes of *Helicobacter pylori* isolated from various acid peptic diseases in and around Lucknow

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The vacuolating cytotoxin and the cytotoxin-associated protein, encoded by *vacA* and *cagA* respectively, are important virulence determinants of *Helicobacter pylori*. A total of 79 clinical isolates of *H. pylori* were characterized for *vacA* and *cagA* genes using specific primers. Fifty-five (69.6%) of 79 strains had *vacA* signal sequence genotype s1a, 13 (16.4%) had type s1b and 11 (14%) had type s2. The *vacA* middle-region types m1 and m2 were detected in 51 (64.6%) and 28 (35.4%) strains. The combinations s1a/m1 (42 [53.2%]), s1a/m2 (13 [16.5%]), s1b/m1 (9 [11.4%]), and s1b/m2 (4 [5.1%]) occurred more frequently than s2/m2 (11 [13.9%)]. No strains with s2/m1 were found ($P < 0.01$). Thirty-one (86.1%) of 36 patients with ulcers harboured type s1a strains, in contrast to 17 (68%) of 25 patients with gastritis. Moreover, all isolates from patients with ulcer, gastritis and gastric cancer were *cagA*$^+$, but only 10 (76.9%) of 13 isolates from patients with portal hypertension carried the *cagA* gene. Strains possessing *vacA* type s1 (37 [66.1%] of 56) were more frequently associated with vacuolating cytotoxicity than type s2 strains ($P < 0.01$). These results indicate that *cagA*$^+$, s1a/m1 type strains are associated with occurrence of peptic ulceration and cytotoxin activities common in and around Lucknow.

HELICOBACTER pylori is a Gram-negative, spiral, microaerophilic bacterium that chronically infects the gastric mucosa of more than half of the world population[1]. It has the unique property to colonize the gastric mucosa and persist in this niche for decades. *H. pylori* infection is a major cause of chronic active gastritis, peptic ulcer diseases[2,3] and an early risk factor for gastric cancer[4]. The pathogenesis of *H. pylori* is influenced by bacterial genotype as well as host response and environmental conditions.

There are specific virulence determinants in *H. pylori* strains, apart from immunological factors in the host, which influence the clinical outcome of infection. One important virulence factor is a vacuolating cytotoxin that induces the formation of intracellular vacuoles in mammalian cells *in vitro*, that leads to cell death[5]. Although the gene that encodes the cytotoxin, designated *vacA*, is present in nearly all strains[6–8], only about 50% of *H. pylori* strains can produce detectable amounts of this cytotoxin[9]. Analysis of *vacA* from different strains has shown that the gene differs in its signal sequence, which could be s1a, s1b, s1c or s2 and in its mid-region sequence, which could be, at least, m1 or m2 (refs 10 and 11).

Another virulence factor of *H. pylori* is cytotoxin-associated (*cagA*) gene; it produces the 128-kDa CagA protein[12]. The presence of *cagA* is associated with duodenal ulceration, gastric mucosal atrophy, and gastric cancer[13]. *cagA* is part of a larger genomic entity, designated the cag pathogenicity island (cag PAI)[14], which contains multiple genes that are related to the virulence and pathogenicity of the *H. pylori* strains. Therefore, the presence of *cagA* can be considered as a marker for this genomic PAI and is associated with more virulent *H. pylori* strains. Nearly all East Asian strains carry the cag PAI independent of disease status[15,16]. In contrast, only one half to two-thirds of the US and European strains carry the cag PAI.

Although *H. pylori* is cosmopolitan, little is known about the geographic distribution of specific *H. pylori* strains, especially in developing countries[17]. Also, data concerning the association between *vacA* genotypes and

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