Vol.4, No.11, 585-594 (2013)
http://dx.doi.org/10.4236/as.2013.411079

A Kunitz trypsin inhibitor from chickpea (*Cicer arietinum* L.) that exerts an antimicrobial effect on *Fusarium oxysporum* f.sp. *ciceris*

Meera Nair*, Sardul Singh Sandhu

Fungal Biotechnology and Invertebrate Pathology Laboratory, Department of Biological Sciences, Rani Durgavati University, Jabalpur, India; *Corresponding Author: meera.nair3@gmail.com

Received 31 August 2013; revised 29 September 2013; accepted 15 October 2013

ABSTRACT

***Fusarium oxysporum* f.sp. *ciceris* (*Foc*) is one of the most important fungal pathogens of chickpea and is regarded as a constant threat in tropical and subtropical countries. In order to correlate Fusarium wilt resistance/susceptibility in *Cicer arietinum* to the presence or absence of trypsin inhibitor (TI) in the crude extract, trypsin inhibitory assay (TIA) and *in vitro* activity of TI against *Foc* were studied. In the present study, a 20 kDa trypsin inhibitor was purified from Fusarium wilt resistant cultivar (*viz.* JG 2001-12) by ammonium sulfate precipitation, dialysis and chromatographies with Sephadex G-100 and Diethyl aminoethyl cellulose (DEAE-cellulose-52) ion-exchange column. Results of pathogenecity assay were found to be in correlation to the trypsin inhibitor assay where the Fusarium wilt resistant cultivar showed high trypsin inhibitory activity (99%) in the presence of trypsin enzyme using both natural and synthetic substrates. Preliminary studies using crude extracts of JG 2001-12 showed a decrease in radial growth of *Foc.* A 45% - 82% reduction in conidium germination at 20 µg·mL^{-1} *Cicer arietinum* trypsin inhibitor (CaTI) concentration was observed, thereby, indicating the use of CaTI in suppression of pathogen and in its deployment through transgenic plants for the management of Fusarium wilt.**

Keywords: *Cicer arietinum*; Fusarium Wilt; Kunitz; Proteinase Inhibitor; Trypsin Inhibitor

1. INTRODUCTION

Cicer arietinum (L.) is a worldwide leguminous crop, which ranks third in the world among pulse crops after peas and beans. Chickpea accounts a substantial proportion of human dietary nitrogen uptake and plays a crucial role in food security in developing countries [1]. Fusarium wilt caused by the Deuteromycetes fungal pathogen *Fusarium oxysporum* f.sp. *ciceris* (*Foc*) Schlechtend. Emend, Synder and Hansen, is a serious devastating disease of chickpea in India, Iran, Pakistan, Nepal, Burma, Spain, Tunisia and Mexico. It is a major pathogen, among 67 reported pathogens, to cause disease in *Cicer arietinum* (L.). Annual yield losses due to this disease estimate a 10% to 15% loss, but Fusarium wilt epidemics can cause a 100% loss under favorable conditions [2]. Persistence of the pathogen in soil and its capacity to survive there for years even in the absence of host [3] renders its control difficult [4]. Soil applications of fungicides are costly and lead to indiscriminate killing of beneficial soil microflora, therefore alternative biocontrol agents or other eco-friendly control strategies are necessary [4].

One of the eco-friendly control measures opted to provide resistance against fungal pathogens is using a proteinase inhibitor (PI) proteins produced in plant tissues, which, act as a defensive mechanism against microorganisms [5]. Plant PIs rely on inhibition of proteases secreted by microorganisms, causing a reduction in the availability of amino acids necessary for their growth and development [6].

C. arietinum (L.) seeds are rich in serine proteinase inhibitors (PIs) which show strong inhibitory activity against trypsin, chymotrypsin or other proteolytic enzymes [7]. In our earlier study, trypsin inhibitor showed antimetabolitic activity against *Helicoverpa armigera* [8]. Accordingly, an attempt has been made to analyze antifungal activity of trypsin inhibitor towards the growth of *Foc* and its influence on hyphal growth. To the best of

our knowledge, this is the first study focusing on the use of *C. arietinum* (L.) trypsin inhibitor (CaTI) on Fusarium wilt control.

2. MATERIALS AND METHODS

2.1. Fungal Cultures

Standard isolates of *Foc* (MTCC # 2087) was procured from Microbial Type Culture Collection and Gene Bank (MTCC), IMTECH (Institute of Microbial Technology), Chandigarh where they were already characterized and classified for their race specificity using conventional method of race identification. The cultures were maintained on Potato Dextrose Agar (PDA) slants with timely sub-culturing and infection to a susceptible cultivar, JG 62.

2.2. Plant Material

Cicer arietinum (L.) cultivar seeds *viz*. JG 2001-12, JG 16 and JG 62 used in this study were obtained from Department of Genetics and Plant Breeding, Jawaharlal Nehru Krishi Vishwavidhyalaya (J.N.K.V.V.), Jabalpur, India. Each plant were grown from single seed, planted in a rectangular trough and maintained under green house conditions. Cultivar JG 62 (pedigree-selection from germplasm), is susceptible to Fusarium wilt.

2.3. Pathogenecity Assay

Seeds of *C. arietinum* (L.) cultivar *viz*. JG 62, JG 2001-12 and JG 16 were pre-germinated and grown in 20 × 10 × 2.25 cm trays filled with peat soil. Freshly prepared spore suspension (1×10^6 spores·mL^{-1}) of *Foc* # 2087 was added individually to the sterile trays containing 7 day old chickpea plants. Seedlings grown in trays with no pathogen (un-inoculated plants) served as control. The pathogenecity assays were conducted in triplicates. Seedlings showing typical disease symptoms such as wilt, xylem discoloration and stunting were recorded for 21 days. Final disease severity data was recorded on 8th week after inoculation. Pathogen was re-isolated from representative diseased plants so to prove Koch's postulates.

2.4. Preliminary Proteinase (Trypsin) Inhibitor Assay

Trypsin inhibitor activity (TIA) of crude extract was determined using both natural (casein) and synthetic oligopeptides substrate (BA*p*NA) by pre-incubating 0.25 µg trypsin (10 mg·mL^{-1} of bovine trypsin in 1 mM HCl) and 50 µl of crude extract (12.5 mg protein) for 15 min in a water bath (D'sco, India) at 37˚C. Natural or synthetic oligopeptide substrate was then added and the reaction was carried out for 30 min at 37˚C. For caseinolytic assay, reaction was stopped by adding 50 µl of 50% (w/v) TCA which was allowed to stand for 15 min at 4˚C. TCA-soluble material was collected by centrifuging the reaction mixture at 2000 × g for 20 min. In BA*p*NA, the reaction was terminated by adding 0.5 mL of 10% acetic acid and absorbance at 410 nm was determined. The percentage at which casein was digested by trypsin was calculated by measuring the absorption of trichloroacetic acid (TCA) filtrate at 280 nm by using the tyrosine standard curve. Similarly, *p*NA (*p*-nitroaniline) was used as a standard for BA*p*NA. Trypsin inhibition (%) was calculated from the difference between untreated (without crude extract) and treated (with crude extract) samples divided by untreated sample reading, multiplied by 100 [9].

2.5. Extraction of Chickpea PIs

The seed extract of resistant cultivar *viz*. JG 2001-12 was prepared as per the method described by Nair *et al*. [8]. Soluble proteins were extracted from milled, defatted and depigmented seeds of chickpea in 10 mM phosphate buffer, pH 7.2 with constant stirring at 4˚C for 4 h. Inhibitor proteins were fractionated with ammonium sulphate (40% - 60% saturation), recovered by centrifugation at 12000 × g for 30 min at 4˚C and later dissolved in a minimal amount of deionized water. The protein was concentrated against 50 mM Tris/HCl buffer, pH 7.5 using dialysis membrane (Sigma Aldrich USA, 12 kDa cut off) and loaded on a Sephadex G-100 (50 cm × 1 cm) equilibrated with chilled 10 mM Tris/HCl buffer (pH 7.5) and eluted with the same buffer. Active fraction showing trypsin inhibitor activity was pooled and concentrated *via* acetone precipitation. Acetone treated aliquots (500 µl) was loaded and left undisturbed for 30 min. The inhibitor was eluted using 0 to 2 M NaCl linear gradients at 4˚C. Total protein was assayed by Lowry *et al*. [10] using bovine serum albumin as a standard.

2.6. Polyacrylamide Gel Electrophoresis Analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the protocol of Laemmli [11] with 4% stacking gel and 12% separating gel using a vertical gel electrophoresis system (GeNei, Bangalore, India), where anion exchange fraction along with standard protein molecular weight marker (Bangalore Genei, India) was loaded to the wells. The gel was run at a constant voltage of 50 V at 4˚C in electrophoresis buffer (25 mM Tris/HCl pH 8.8, 192 mM glycine and 0.1% (w/v) SDS) following which, the entire gel was washed with distilled water and stained with Commassie brilliant blue stain (Calbiochem®) for 30 min with constant shaking. The gel was later destained with

methanol:glacial acetic acid:distilled water (v/v) (3:2:15) for one hour giving three changes of destain.

2.7. In-Gel Assay of Proteinase Inhibitor

Gelatin zymography were performed according to Le and Katunuma [12] with resolving 10% acrylamide solution containing 0.1% gelatin. Ion exchange chromatography fraction equivalent to 40 µg protein were mixed with equal volume of sample buffer containing 25 mM Tris/HCl (pH 6.8), 20% glycerol, 4% SDS and 0.02% bromophenol blue was loaded along with 80 µg of soybean trypsin inhibitor (Calbiochem®; 10 mg·mL^{-1} stock solution in Tris/HCl pH 8.2) which was used as a marker. Electrophoresis was performed at a constant voltage of 50 V at 4˚C in electrophoresis buffer (25 mM Tris/HCl, pH 8.8, 192 mM glycine and 0.1% SDS). The gel was washed for 30 min at room temperature with 0.1 M Tris-HCl at pH 7.2 containing 2.5% Triton X-100, followed by distilled water for 20 min. Subsequent incubation at 37˚C for 3 h with reaction buffer containing 10 mg·mL^{-1} trypsin solution (10 mg trypsin in 0.1 M Tris/HCl, pH 7.5) and 0.02% NaN_3 followed by Coomassie Brilliant Blue R250 (CBB) staining for 1 h and destaining produced blue bands against a clear background.

2.8. Determination of Proteinase (Trypsin) Inhibitor Activity at Different Stages of Purification Steps

The proteinase inhibitor activity on natural and synthetic chromo(non-chromo)genic substrates was determined in a reaction mixture containing 50 mM Tris/HCl buffer (pH 7.5), 0.25 µg trypsin (EC 3.4.21.4) (10 mg·mL^{-1} of bovine trypsin in 1 mM HCl), different concentration of all three ammonium sulphate cut off (100 µg), gel filtrate fraction (100 µg) and ion exchange fractions. The anion exchange fractions (43 µg, 86 µg, 430 µg protein) with 100 µl casein, 40 µl BA*p*NA (10 mg·mL^{-1} in dimethyl sulfoxide); 0.23 mM BAEE in 67 mM potassium phosphate buffer (pH 7.5), 12.5% azocasein (w/v) in 50 mM Tris, 5 mM $CaCl_2$ (pH 8.0). Controls with a trypsin enzyme (100˚C, 5 min) were run in parallel. The amount of *p*-nitroaniline (*p*NA) released was determined by measuring the change in absorbance at 410 nm using *p*NA calibration curve. Proteinase inhibitor (TI) activity was also determined using a non-chromogenic substrate N-benzoyl-L-arg ethyl ester (BAEE) according to Schwert and Takenaka [13].

In azocasein, the reaction was terminated by adding 200 µl of ice-cold 5% TCA to 500 µl of reaction mixture. The reaction mixture was then placed at room temperature for 10 min and centrifuged at 3,000 × g for 10 min, the supernatant (500 µl) was later aspirated and 1500 µl of 0.5M NaOH was added. The absorbance was recorded at A_{428} [14].

2.9. Screening of Fungitoxic Activity

The *in vitro* fungitoxic activity of crude extract of fusarium wilt resistant cultivar (*viz.* JG 2001-12) and fusarium wilt susceptible cultivar (*viz.* JG 16) was performed by mycelial disc method as described by Tremacoldi and Pascholati [15]. The mycelial disc was placed onto PDA medium, previously flooded with filtered (0.2 µm, Millipore) crude extracts in 25 µl, 500 µl, 1000 µl and 2000 µl volumes. The experiment was performed in triplicate for each concentrations and controls (with no crude extract added) under strict aseptic conditions. The fungitoxic activity of each concentration was expressed in terms of the mean of colony diameter in cm (mean value ± standard deviation) produced by respective extract at the end of the incubation period (7 d). Gel filtration fraction (Fraction A) was also tested against mycelia growth of *Foc* isolates at 10, 100 and 200 µg of protein (as described above).

2.10. Effect of Anion Exchange Fractions on the Hyphal and Conidial Growth of *Fusarium oxysporum* f.sp. *ciceris*

The effect of purified CaTI protein (anion exchange fractions) on hyphal growth and conidial germination was assayed. Conidial suspension was prepared by flooding the cultures with sterile distilled water containing 0.05% (v/v) Tween-80 which was later filtered through four layers of sterilized cheesecloth to remove adhering mycelia. The spore concentration was adjusted to 10^5 conidia per mL with the aid of Neubauer hemocytometer. Conidia were allowed to germinate and grown in the presence of CaTI at 0, 5, 10, 15, 20 µg·mL^{-1} concentrations in 6-well plate at 25˚C in darkness with a positive control and negative control plates containing heat inactivated CaTI and phosphate buffer. For each treatment, hyphal length was measured from 40 randomly selected hyphae and the mean hyphal length was used for comparison using a light microscope equipped with an ocular micrometer after 12 h and 24 h of incubation. As *Foc* is multicelled therefore; a conidium was considered germinated, if hyphae were visible for at least one of the cells. Each treatment contained three replicates and the experiment was repeated twice.

2.11. Statistical Analysis

The results were subjected to analysis of variance (ANOVA) and the significance of differences among means was determined by using t-test using the MS Excel program where p values <0.05 were considered significant. All experiments were performed in triplicate unless stated otherwise. Comparisons were made for all

parameters within the amount of inhibitor used as well as the hours of incubation during antimicrobial activity on *Foc*.

3. RESULTS AND DISCUSSION

3.1. Pathogenecity Assays

Chickpea plants *viz*. JG 62, JG 2001-12 and JG 16 were inoculated with fungal spores of *Foc* individually where un-inoculated plants served as control. Upon infection, JG 62 and JG 16 showed wilting symptoms by 10 days after inoculation (dai), while no wilting was observed in JG 2001-12 cultivar even by the 8th week. **Figure 1** shows the chickpea cultivars, where JG 16 exhibit wilting symptoms whereas JG 2001-12, display no wilting symptoms.

3.2. Preliminary Proteinase (Trypsin) Inhibitor Activity

The inhibitory activity of the crude extract (*viz*., JG 2001-12 and JG 16) against bovine trypsin was determined using natural and synthetic substrate *viz.* casein, BA*p*NA respectively.

Caseinolytic activity: Crude extract of JG 2001-12 and JG 16 was used to determine the proteinase inhibitory activity where the specific activity of trypsin was found to be 227 ± 0.5 nmol·tyr·min^{-1}·mg^{-1} protein in absence of crude extract (trypsin inhibitor). The crude extract of Fusarium wilt resistant cultivar (*viz.* JG 2001-12) displayed a 99% trypsin inhibition with 0.7 ± 0.4 nmol·tyr·min^{-1}·mg^{-1} protein. The caseinolytic activity of Fusarium wilt susceptible cultivar (*viz.* JG 16) showed a 12% inhibition with 200 ± 0.5 nmol·tyr·min^{-1}·mg^{-1} protein TIA (**Table 1**).

BA*p*NA: Trypsin activity was rapidly inhibited (99.4% inhibition) by the crude extract of *C. arietinum* fusarium wilt resistant cultivar (*viz.* JG 2001-12), indicating the presence of trypsin inhibitor with 1.0 ± 0.3 µmol *p*NA min^{-1}mg^{-1} protein TIA against bovine trypsin (**Table 1**).

3.3. Purification of Cicer Arietinum Trypsin Inhibitor (CaTI)

The trypsin inhibitor from JG 2001-12 seed crude extract was identified by trypsin inhibitory activity (TIA) assays using trypsin as the enzyme at preliminary identification and purification steps. The purification results are presented in **Figure 2** and **Table 2**. The proteinase inhibitor activity was revealed in 40% - 60% ammonium sulphate cut off (**Figure 3**). Chickpea proteinase (trypsin) inhibitor was isolated by dialysis, desalting in Sephadex G-100 and purified using DEAE-cellulose column eluted with 0.1 M NaCl. Upon size-exclusion chromatography

Table 1. Trypsin Inhibitory activity (TIA) of crude extracts of fusarium wilt resistant (JG 2001-12) and susceptible (JG 16) cultivars.

		Casein		BA*p*NA	
		TIA*	TI$^{\#}$	TIA*	TI$^{\#}$
	Control	227.6 ± 0.5	0	172.01 ± 0.5	0
	Crude extract of cultivar				
1)	JG 16	200^{b} ± 0.5	12	145.2^{a} ± 0.3	15.5
2)	JG 2001-12	0.7^{a} ± 0.4	99	1.0^{b} ± 0.3	99.4

^{a,b}Means with different superscripts in a column differs significantly ($p < 0.05$). *TIA = nmol tyr min^{-1}mg^{-1} protein; **TIA = µmol *p*NA min^{-1}mg^{-1} protein; TI$^{\#}$ = Trypsin Inhibition (%).

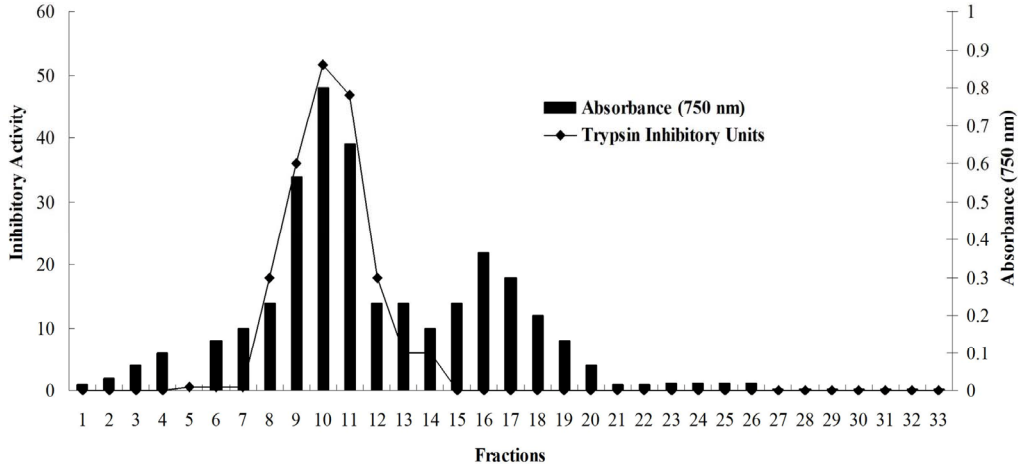
Table 2. Purification performance of Trypsin Inhibitor from JG 2001-12 cultivar.

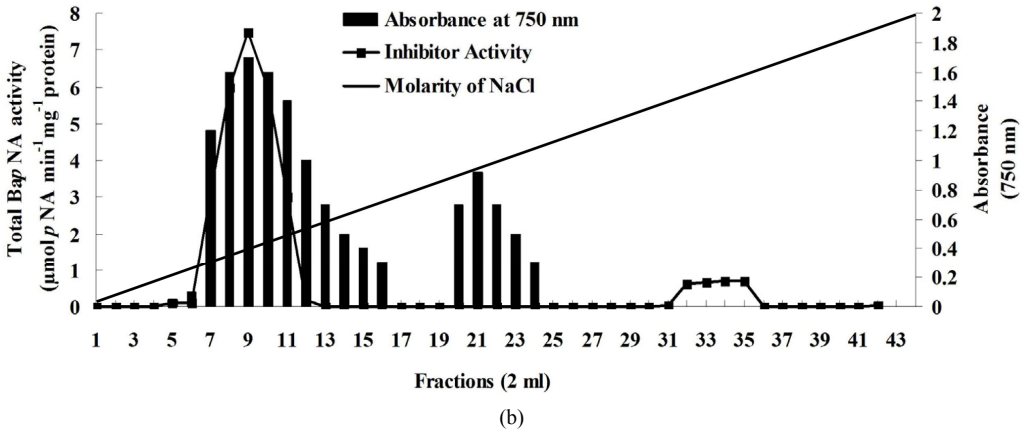
Sample	Total protein (mg)	Specific Activity (µM *p*NA min^{-1}mg^{-1})	TIA*	Yield (%)	Purification Fold
Crude Extract	258	2.32	44.5	100	1
Sephadex G-100	9	72.54	86.7	25	57.1
DEAE-cellulose-52 column	0.1	140	94.4	10	65.4

*TIA = Trypsin Inhibitory activity.

Figure 1. Fusarium wilt **s**usceptible cultivar (JG 16) and resistant (JG 2001-12) showing wilting and chlorosis.

on Sephadex G-100 column, two protein peaks were obtained where proteinase inhibitor was detected in the first peak (**Figure 2(a)**) which consisted of three fractions (fraction 9, 10, 11 called as Fraction A). Fraction A showed maximum trypsin inhibitory activity against trypsin enzyme (**Figures 4(a)**, **(b)**). Further, the trypsin inhibitor was retained by anion exchange column-DEAE-cellulose column and eluted in fractions of 0.1 M NaCl (**Figure 2(b)**). Taken together, approximately 10% recovery and 65.4 fold increase in specific activity resulted (**Table 2**). Fractions of size exclusion chromatography, anion exchange chromatography were employed on SDS-PAGE and reverse zymography (using gelatin as substrate) to verify the purity of trypsin inhibitor and





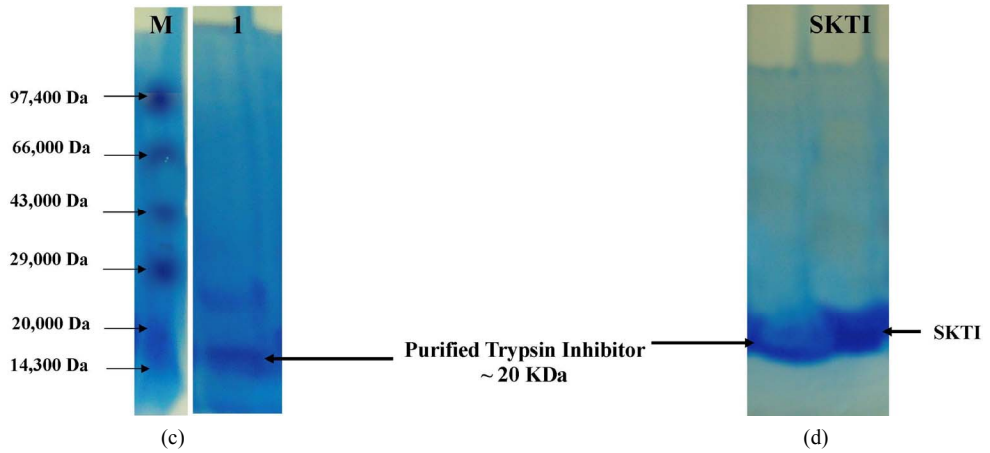


Figure 2. Purification of Trypsin inhibitor from JG 2001-12 seeds. (a) Separation using Sephadex G-100 column chromatography of trypsin inhibitor from seeds of *Fusarium* wilt resistant *Cicer arietinum* L. cultivar *viz.,* JG 2001-12. (b) Purification of higher trypsin inhibitory region (fraction 9, 10, 11) identified after Sephadex G-100 chromatography using DEAE-cellulose ion exchange chromatography column. Line across the chromatography represents the linear 0-1 M NaCl gradient employed to elute adsorbed proteins. (c) SDS-PAGE gel of trypsin inhibitor purified using ion exchange chromatography with standard molecular weight marker (M). (d) In gel assay detection of trypsin inhibitory activity. Standard soybean kunitz trypsin inhibitor (SKTI) and Purified trypsin inhibitor from JG 2001-12 seeds.

estimate its molecular mass. Trypsin inhibitor was resolved by SDS-PAGE and in-gel digestion of gelatin in zymograms where both gels showed one band corresponding to M_r ~20 kDa (**Figures 2(c)** and **(d)**).

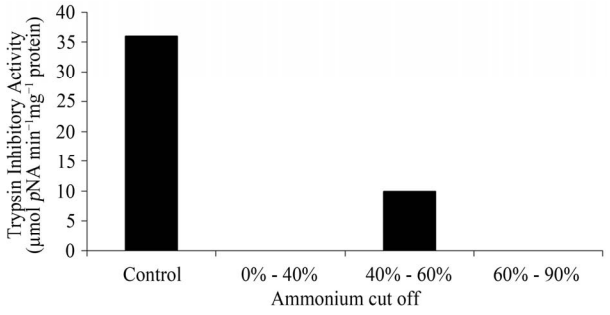


Figure 3. Trypsin Inhibitory Activity (TIA) in different ammonium sulphate cut offs of protein homogenate of JG 2001-12 cultivar in comparison to control (with no crude extract) using BA*p*NA substrate.

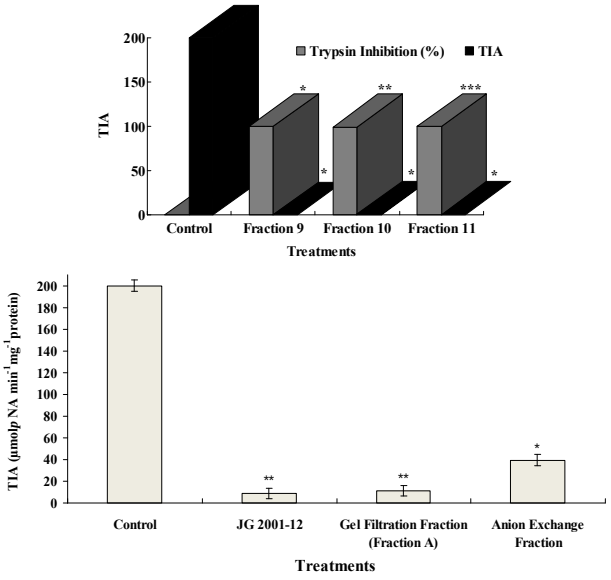


Figure 4. Trypsin Inhibitory Activity (TIA) Assay.

3.4. Trypsin Inhibitor Activity Assay

Trypsin inhibitor activity assay (TIA) of purified trypsin inhibitor at different purification steps using synthetic substrates (BA*p*NA, azocasein, BAEE) were carried where the reliability of the reaction conditions was determined in triplicate sets. The TIA using BA*p*NA substrate, at different concentration of CaTI protein [(5 µL) (43 µg), 10 (86 µg), 15 µL (430 µg)] showed a progressive reduction in trypsin activity, with increase in concentration of CaTI protein. The inhibitory activity was increased from 86.2%, 97.2% to 98.61% with protein concentration (**Figure 5**). Similarly, in azocasein and BAEE substrate, absorbance at 428 nm and 253 nm was found to be high in control (with no CaTI) with respect to the proteinase activity in the presence of trypsin inhibitor in fusarium wilt resistant cultivar crude extract *viz*. JG 2001-12. The trypsin activity was calculated in units where, one unit of trypsin was defined as the amount that increased A_{428} by 0.01/min under the assay conditions.

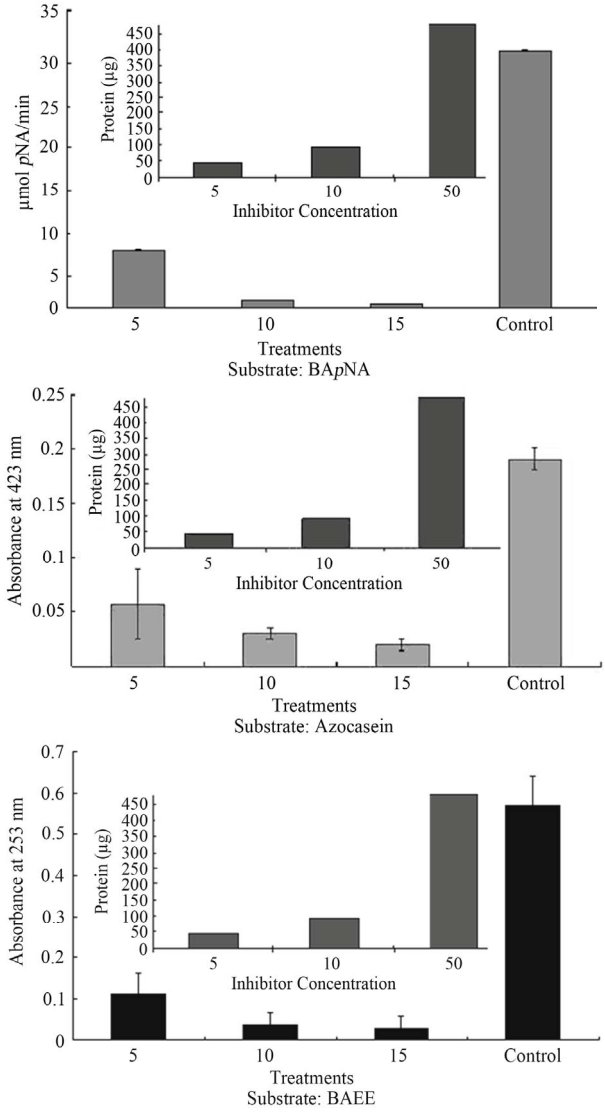


Figure 5. Effect of different concentration of trypsin inhibitor on activity of trypsin using synthetic substrates (BA*p*NA, Azocasein, BAEE) as described in Materials and Methods. Error bars represent standard deviations. Inset, showing the protein concentration at different volume of trypsin inhibitor.

Trypsin inhibitory units were calculated from the number of trypsin units under similar conditions in the presence of trypsin inhibitor. 50 µl of inhibitor (320 µg equivalent protein) was used as the optimum concentration so to inhibit trypsin activity. It was found that after 30 min of incubation, there was no increase in the absorbance at 428 nm and 253 nm, suggesting an inhibition in trypsin activity (**Figure 5**). Our results demonstrated no variation in proteolytic activity of trypsin on natural and synthetic substrates in presence and absence of trypsin inhibitor. The results indicated that the crude extract of fusarium wilt resistant cultivar (*viz*. JG 2001-12) inhibit proteolysis of natural and synthetic substrates in the presence of bovine trypsin. The method we adopted here

does not require ampholytes, so it is a cheaper and more convenient method. In the present study, a comparison on the trypsin inhibitor activity was carried out using different natural and synthetic substrates where, the TIA results were in conformity to Spelbrink *et al.* [16]. Earlier reports also suggest the use of both natural and synthetic substrates in determination of antitryptic activity as natural substrates are more difficult to displace from the active site to form the enzyme inhibitor complex while synthetic substrate are accurate in determining the inhibitor content of the materials [17] thereby providing a value with physiological relevance [16].

3.5. Effect of Crude Extract of Seeds on the Mycelial Growth of *Fusarium oxysporum* f.sp. *ciceris*

Studies have shown that an increasing level of proteinase inhibitor correlates to an increase in resistance against pathogens [18]. In many plant species, response to infection by pathogenic bacteria, viruses and fungi or to various abiotic stresses is accompanied by the synthesis of a variety of proteins termed as Pathogenesis related (PR) proteins [19], which have been isolated from both dicots and monocots [20]. Preliminary short report by Niderman *et al*. [20] has described a direct fungicidal activity of PR-1 protein from tomato. Similar results with PR-2, protein P14 against fungal pathogens have also been elucidated. In the present communication, crude extract of *C. arietinum* (L.) seed was preliminarily screened so to observe the effect of trypsin inhibitor on mycelial growth of *Foc.* The amount of proteins in the crude extract was equivalent to 3.5% in relation to the fresh weight of the seeds. The categorization of crude extracts as per TIA assay was also in accordance to the pathogenicity assay. Test plates containing crude extract of JG 2001-12, exhibited a colony diameter of 4.3 ± 0.04 cm whereas control plates exhibited a colony diameter of 9.0 ± 0.05 cm (**Figure 6(b)**). Mycelial growth was found to be static with a decrease in colony diameter in presence of JG 2001-12 crude extract whereas susceptible cultivar, JG-16, and control plates where no crude extract was added showed no inhibition. Observations suggest CaTI to be antifungal, as JG 2000-12 showed significant antifungal activity w.r.t JG 16 cultivar, whose growth was analogous to control plates.

3.6. Effect of gel filtration fractions (showing trypsin inhibitory activity) on the mycelial growth of *F. oxysporum* f.sp. *ciceris*

Results showed a decrease in the growth of mycelia as the fraction showing trypsin inhibitor challenged the growth of *Foc* isolate # 2087 (3.0 ± 0.04 cm) as compared to the control where the growth of the isolate was found to be normal (9.0 ± 0.04 cm). Gel filtrate fractions (fraction "A") showed a significant inhibition in fungal growth, indicating presence of trypsin inhibitor which led to inhibition in radial growth at protein concentration of 200 µg (**Table 3**). Thus, demonstrating trypsin inhibitor's defensive role in inhibiting fungal growth. Using gel filtrate fraction, a 100-fold increase in inhibitory activity was observed over the crude inhibitor preparation against *Foc* (**Table 3**).

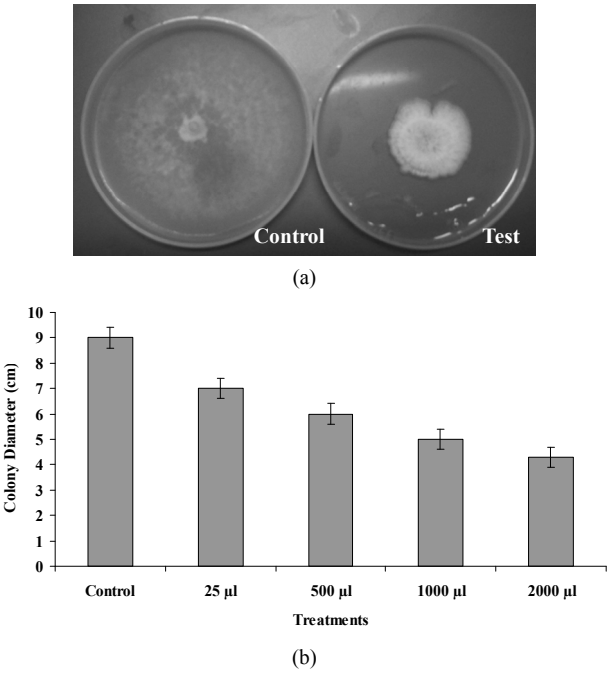


Figure 6. Effect of crude extract on the growth of Fusarium oxysporum f.sp. ciceris. (a) In vitro plate assay of crude extract from JG 2001-12 (T) on growth of Foc with respect to the control (C). (b) Effect of crude extract from JG 2001-12 on the growth of Foc

3.7. Effect of Anion Exchange Fractions (Showing Trypsin Inhibitory Activity) on the Conidial Growth and Hyphal Length of *F. oxysporum* f.sp. *ciceris*

As the volume of fractions achieved after anion exchange was small therefore, the purified trypsin inhibitor effect on conidial growth and hyphal length of *Foc* was observed. For most isolates, conidium germination decreased dramatically with increasing trypsin inhibitor (CaTI) concentration. Conidium germination was reduced to 50% at a CaTI concentration of 20 $\mu g \cdot mL^{-1}$. After 12 h of incubation, a significant difference was observed both visually (**Figure 7**) and quantitatively (**Figure 8**) in fungal growth; as condium germination was found to be less as compared to the control (10% PDB un-amended with CaTI). In 10% PDB amended with

Table 3. Effect of gel filtrate fraction "A" on growth of *Fusarium oxysporum* f. sp. *ciceris* at different concentration of protein (µg).

	Treatment	Colony diameter (cm)
1)	Control	9.0 ± 0.04
2)	10 µg	5.0 ± 0.2
3)	100 µg	4.0 ± 0.25
4)	200 µg	3.0 ± 0.04

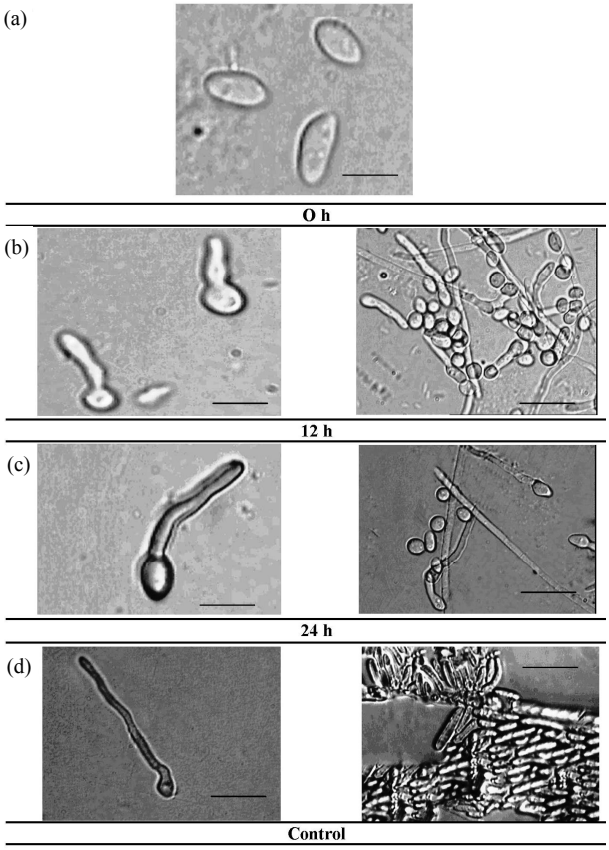


Figure 7. Inhibition of *Fusarium oxysporum* f.sp. *ciceris* conidia germination. Ion exchange chromatography fractions are mixed with PDB and incubated at 37˚C. Bar = 10 µm. (a) 10% potato dextrose broth (PDB) alone at 0 h; (b) PDB amended with trypsin inhibitor (20 µg·mL^{-1}) at 12 h; (c) PDB amended with trypsin inhibitor (20 µg·mL^{-1}) after 24 h; (d) Control, with no trypsin inhibitor.

CaTI (15 µg·mL^{-1}), hyphal growth was observed to be 25 µm as compared with 200 µm in 10% PDB (control). At the end of 24 h of incubation, PDB amended with CaTI (15 µg·mL^{-1}) showed an increase in hyphal growth from 25 µm to 52 µm, whereas there was a drastic increase in germination and hyphal growth of the mycelia (340 µm) in 10% PDB (control).

At the end of 24 h of incubation, the hyphal length in the PDB amended with CaTI at 20 µg·mL^{-1} remained

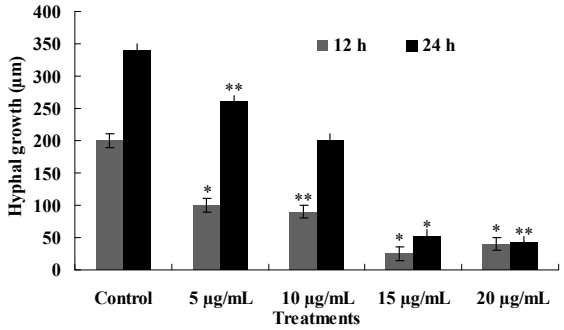


Figure 8. Fungal hyphal growth (µm) with different treatments of trypsin inhibitor fractions of anion exchange column chromatography as described in Materials and Method.

nearly the same as that at 12 h with little variation (*i.e.* at 12 h the hyphal growth was found to be 40 µm and after 24 h the hyphal growth was 42 µm). The results obtained using conidial germination and hyphal growth was in correlation to each other as the results showed inhibition in the presence of trypsin inhibitor were consistent (**Figures 7** and **8**). These studies were similar to Chen *et al.* [21] reports, which showed that the resistance of certain corn genotypes to fungal infection is related to the action of trypsin inhibitor, which is due to the lowering in the production and activity of fungal alpha-amylase, which in turn reduce the availability of simple sugars for fungal growth. Similar studies on the inhibition of spore germination and mycelium growth of *Alternaria alternata* by buckwheat trypsin/chymotrypsin was observed [22]. Likewise, cysteine proteinase inhibitors from pearl millet also inhibited the growth of many pathogenic fungi including *Trichoderma reesei* [23]. In addition, the antifungal effect of trypsin inhibitor was observed in wheat kernel [24], corn [21], barley [25] and cabbage [26].

Our results were in corroborance with Chen *et al*. [21], Huang *et al*. [27], Revina *et al*. [28] who described the use of resistant TI in inhibiting germination and hyphal growth of plant pathogenic fungi. Collating the present and previously reported data, it appears that *Cicer arietinum* trypsin inhibitor (CaTI) act as an antifungal agent since *in vitro* activity of CaTI showed a 45% - 82% suppression of conidial germination and hyphal growth, thereby showing its effectiveness against *Foc*.

As *Foc* survives in soil, therefore use of TI containing *Cicer arietinum* L. (CaTI) would be an easy approach in order to reduce the use of chemicals/fungicides to combat Fusarium wilt. In the present study, a sustainable approach in controlling Fusarium wilt was utilized *via* the use of trypsin inhibitor as implementation of cultural, physical, biological and chemical measures minimize economic risks to consumers and the environment. To sum up, CaTI provide a defensive property to host plant

as a cultivar containing trypsin inhibitor showed resistance against Fusarium wilt. In this context, future research to identify eco-friendly tools for the biological control of pathogens and further studies are warranted to confirm its biotechnological potential against other pathogens inflicting *Cicer arietinum* (L.).

3.8. Abbreviations and Acronyms

BAEE, N-benzoyl-L-Arg ethyl ester; BA*p*NA, N-*α*-Benzoyl-D, L-arginine *p*-nitroanilide hydrochloride; CLA, carnation leaf agar; CaTI, *Cicer arietinum* Trypsin Inhibitor; *Foc*, *Fusarium oxysporum* f.sp. *ciceris*; PDA, Potato Dextrose Agar; PDB, Potato Dextrose Broth; *p*-NA, *para*-nitroaniline; TI, Trypsin Inhibitor; TIA, Trypsin Inhibitor Activity; TCA, Trichloroacetic acid.

4. ACKNOWLEDGEMENTS

The authors would like to thank the Head of the Department of Biological Sciences, Rani Durgavati University, Jabalpur (India) for laboratory facilities and University Grant Commission Project (Govt. of India), New Delhi for financial assistance vide project no. UGC (32-370/2006) (SR) dated. 24/04/07. Authors also acknowledge JNKVV, Jabalpur, India for providing the seed material.

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