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Biochemical and Molecular Characterization of Protease from *Arthrobotrys conoides* And *Duddingtonia flagrans*

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ABSTRACT

Proteases form nematophagous fungi are most important extracellular hydrolytic enzymes paying a central role in cuticle degradation. In the present study, protease from two nematode-trapping fungi, *A. conoides* GenBank accession no. JX979095 and *D. flagrans* JX979096 were studied at biochemical and molecular level. Crude protease of *A. conoides* showed maximum activity at pH 7 and temperature 50°C while protese of *D. flagrans* was functioning best at pH 8 and temperature 55°C. 726 and 716 bp serine protease gene fragment from each fungus was amplified and sequences were submitted to GenBank under the accession No. KC769585 and KC862257. Phylogenetic relationships showed homology with serine proteases of other nematode-trapping fungi. Moreover protease activity in culture broth of fungi inoculated with nematodes was increased. Biochemical assay demonstrated 2.14 and 6.4 fold increase in protease activity in induced culture of *A. conoides* and *D. flagrans* respectively. Real Time-PCR assay confirmed 4.27 and 23.8 fold increase in serine protease gene activity in *A. conoides* and *D. flagrans* respectively. Thus like other nematode trapping fungi, serine protease of these two fungi plays important role in virulence against nematodes.

Key words: *Arthrobotrys conoides, Duddingtonia flagrans, Nematophagousfungi, Real Time-PCR, Serine protease.*

[I] INTRODUCTION

Nematode trapping fungi have attracted attention many researchers across the globe due to its attractive life style and its possible use as biocontrol agent for plant and animal parasitic nematodes [1,2,3]. This group of fungi kill nematodes by systematic way of infection [4].

Firstly, fungi recognise the presence of nematodes, adheres to the host surface and capturers it, this is followed by penetration through host cuticle, which ultimately lead to death of prey [5,6]. Nematode cuticle is mainly made up of collagen and other proteinous material [7,8]. Many studies support that extracellular hydrolytic enzymes, i.e. collagenases, chitinases, and proteases are the most important extracellular enzymes for pricking the nematode cuticle or egg shell [9,10,11]. Among these, proteases have been extensively studied and are found an important player in degradation of host. [12,13,14,15]. Amongst the different nematode trapping fungi, *Duddingtonia flagrans* is a potential biocontrol agent against the gastroenteritis causing nematodes in small ruminants [16,17,18,19], While *Arthrobotrys conoides* is a potential candidate for plant parasitic nematodes [2]. However, the serine protease of these two fungi is till date not studied in detail. There are very few reports on the study of serine protease from both these two fungi [14,15,20,21,22].

Further it is established that, the presence of nematodes induces nematophagous fungi for its predacious life style [23]. So it is not surprising that after trapping nematodes, these fungi must secret hydrolytic enzymes in order to penetrate the host cuticle. The up regulation of serine protese gene in the presence of nematodes is reported in *Monacrosporium haptotylum* [24] and *Arthrobotrys oligospora* [25]. However, the induction of protease activity in *Arthrobotrys conoides* and *Duddingtonia flagrans* in the presence of nematodes is yet unknown. Real Time PCR also called quantitative PCR or qPCR is a method of choice to study expression of gene [26]. In the present study, we have characterized serine proteases form our two isolates of nematode trapping fungi, *A. conoides* (RPAN10) and *D. flagrans* (RPAN12). Furthermore, induction of serine protease gene in the presence of plant parasitic nematodes (*Meloidogyne sp*.) was evaluated using biochemical enzyme assay and was further confirmed through Real Time PCR.

[II] MATERIAL AND METHODS

2.1 Fungal isolates and its growth conditions

Two isolates of nematode trapping fungi *A. conoides*, (JX979095) and *D. flagrans* (JX979094) that were used in the present work were originally isolated from agriculture soils in Anand district, Gujarat [27]. Fungi were maintained on 1.7 % CMA, pH 7.0 by inoculating and incubating at 28 ± 1°C for 7 days and stored at 8-10°C.

2.2 Protease enzyme production

For protease enzyme productions, medium described by Braga et al., 2011 [14] pH7.5 was used for both the fungi. Spore suspension of fungi was prepared by flooding 5 mL sterile distilled water on 7 days old culture grown on CMA (Hi-media) at 28±1 ºC. 1mL of spore suspension was inoculated to 50mL of medium in 250mL Erlenmeyer flask in duplicate and allowed grow at 28±1ºC, 125rpm on rotary shaker for 6 days. On 7th day, culture broth was centrifuged at 10000rpm for 10 minutes at 4°C. Protease activity was estimated using 1% casein as a substrate by method described for protease assay by Sigma Aldrich (SSCASE01.001, 1999) using Folin & Ciocalteu's phenol reagent. Reaction mixture contains 5mL 1% casein prepared in potassium phosphate buffer pH 7.5 and 1mL of crude enzyme and incubated at 37°C for 30min. After 30 min 5mL of 110mM trichloroacetic acid was added to stop the reaction. Folin & Ciocalteu's phenol reagent (F-C) was used for color development and absorbance was measured at 660nm. L-Tyrosine (Hi-Media, India) was used standard. One unit was defined as the amount of enzyme that releases 1µM of tyrosine/ml/minute under the assay condition and total protease activity was calculated. Uninoculated flasks were used as control.

2.3 Characterization for protease enzyme

For determining optimum pH, 1% casein was prepared in potassium phosphate buffer having different pH Viz., 4,5,6,7,8 and 9. Optimum temperature was determined by incubating reaction mixture at pH 7.0 at different

temperatures Viz., 15,25,30,37,50,55,60 and 75°C.

2.4 Molecular characterization of serine protease gene

Genomic DNA was isolated from both the fungi and the serine protease gene was amplified using sequence specific primers designed from the reference sequence GenBank accession no. AY859782 using software Primer3 (http://primer3.wi.mit.edu/). Primers that were used are DfsF 5'GACCGTATCTCCCACGAGGA3' (F), DsfR 5'TGCCGTCAGAGTCGGTATTG3' (R) for *D. flagrans* and AcsF 5'GGTGGTTTCGACAAGGCAAC3' (F) and AcsR 5'TGTTGTTGCTGTCAATGGCG3' (R) for *A. conoides*. Primers were synthesized from Sigma (India). 25µl PCR reaction mixture contains 1 µl (50-70ng) DNA, 1µl each primer (10µM), 2.5 µl dNTPs mix (2.5mM each), 2.5 µl 10X TaqA Assay buffer, 0.2µl Taq DNA polymerase (5U/µl) and 16.8 µl MiliQ water. Taq DNA and dNTPs were parched from Genei, Bangalore. Amplification of the target sequence was carried out in Thermo cycler (Corbett, Korea) with cycling profile of pre-PCR at 94º C for 5 min, followed by 35 cycles of denaturation at 94º C for 1 min, primer annealing at 58º C for 1 min, and elongation at 72º C 1 min. A post run of 5 min at 72º C was carried out after the final cycle. PCR products were electrophoreses at 100V in 1.5% agarose gel with 100bp DNA marker and visualized in UV transiluminator. Amplified products were sent to Eurofins Genomics India Pvt. Ltd., Bangalore for sequencing from both directions using forward and reverse primer used in PCR reaction. Both the sequences were analyzed by Bioedit and CodonCode Aligner softwares to make consensus sequence and identified by BLASTn programme at NCBI database. Sequences were searched for possible ORF by StartORF (http://star.mit.edu/orf/runapp_html.html) with the parameter minimum ORF length 100. Phylogenetic analyses with 18 different serine protease gene sequences of nematophagous fungi having different infection procedure were performed by MEGA5 software [28] (Tamura et al., 2011). Serine protease gene of *Metarhizium anisopliae* (FJ659175) was used as outward. The evolutionary history was inferred using the Neighbor-Joining method [29]. The bootstrap consensus tree inferred from 1000 replicates [30] is taken to represent the evolutionary history of the taxa analyzed. Further the amino acid sequences of the predicted ORF were allied to 10 different serine protease amino acid sequences of different nematophagous fungi by Muscle alignment using CLC genomic workbench v7.0.1 (CLC Bio, Denmark). The nucleotide sequences were deposited in GenBank.

2.5 Induction of fungi for protease activity

Both the fungi were allowed to grow in 50ml half strength potato dextrose medium (PDB) (Hi-media) at 28°C for 5 days under static condition so as to form a mycelial mat. We used half strength medium and provide static condition because nutrient deprived condition enhances nematophagous fungi for predacious life style. On 6th day, 2ml (~2000 nematodes) of *Meloidogyne sp*. nematodes were added to flasks containing mycelial mat and further incubated for 24h. Flasks devoid of nematodes were serves as control (un-induced). After 24h, culture media were filtered through sterile Whatman filter paper, pre washed with DEPC treated water, in laminar hood to prevent any contamination. Spent broth was estimated for protease activity as mentioned above and mycelia were used for RNA extraction.

1.6 Conformation of induction of serine protease gene through Real Time PCR

Immediately after filtering, approximately 200mg of mycelia from each induced and control flasks were observed in light microscope under 4X magnification. Any nematodes that were present along with mycelia were carefully removed using sterile spine. Total RNA was extracted using TRI reagent (Sigma, INDIA) followed by chloroform and isopropanol precipitation. Total RNA was treated with DNaseI (Fermentas, USA) to remove any DNA

traces. RNA was reverse transcribed to cDNA using MuLV Reverse Transcriptase (Ferments, USA). Primers for RT-PCR were designed from the predicted ORFs of both the sequences, using the software CloneManager v9. Primer that were used for RT-PCR were 5'TATCGCCGGGAAGACCTATG3' (F) and 5'CTGGCAACGGAGAAGTTTGG3' (R). Beta-tubulin specific primers APKbtFR1 5'GGTAACCAAATCGGTGCTGCTTTC3' (F) and APKbtRE1 5'ACCCTCAGTGTAGTGACCCTTGGC3' (R) were used as endogenous control to normalize RNA concentration. 20.0 µl RT-PCR reaction mixture contains 2.0 µl cDNA, forward and reverse primers 0.75 µl each (10pmol), SYBR master mix 10.0µl (Life Technologies, USA) and 6.5µl Nuclease free water. All RT-PCR reactions were performed in triplicates in optical 96 well plates using ABI prism 7500 Fast Real Time PCR system. Thermal cycling condition comprised of initial denaturation at 95 °C for 10 min followed by 45 cycles of denaturation at 95 °C for 15 sec, primer annealing and extension at 60 °C for 1 min. At the end of each run, a melt-curve analysis was perform (95 °C for 15 sec, 60 °C for 1 min and increase of 0.5 °C/5 sec until 95 °C) was performed to assess the specificity of the amplification. Relative expression or fold of expression was calculated using $2^{-\Delta\Delta C}t$ [31].

[III] RESULTS AND DISCUSSION

3.1 Protease enzyme production and its characterization

Both the isolates produced proteases after 6 days of incubation. Total protease activity was 20.49 U and 24.64 U for *A. conoides* and *D. flagrans* respectively. Temperature profile showed that proteases from both the fungi are stable between 50-60°C. Maximum protease activity from *A. conoides* was found at 50°C this was analogous to Wang et al., 2007 who reported maximum activity at 53.2°C for serine protease of *A. conoides*. Similarly the proteases from *D. flagrans* showed maximum activity at 55°C. Sharp decline in protease activity was observed between temperature 60-75°C and from 15-30°C (Figure-1). This shows that proteases from both the isolates are effective well at a temperature between 50-55°C.

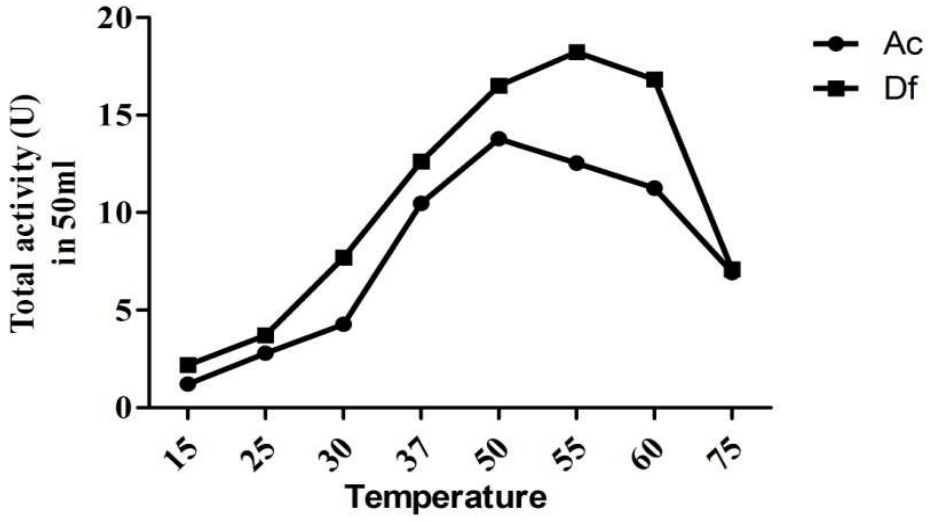

Fig. 1. Thermo stability of crude proteases from Ac-*Arthrobotrys conoides* and Df- *Duddingtonia flagrans*.

Among the pH, proteases from *A. conoides* are found to most active at pH 7, while in the case of *D. flagrans*, it showed highest activity at pH 8. This showed that proteases of *D. flagrans* are alkaline proteases while in case of *A. conoide* it is neutral proteases. This was again comparable to [22,14]. In general proteases from both the fungi remained stable between pH 6-8 (Figure-2).

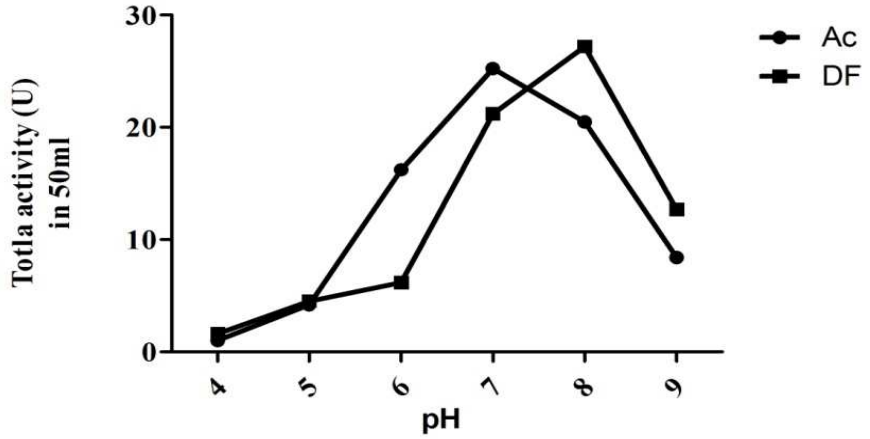

Fig. 2. Effect of pH on crude proteases from Ac-*Arthrobotrys conoides* and Df- *Duddingtonia flagrans*.

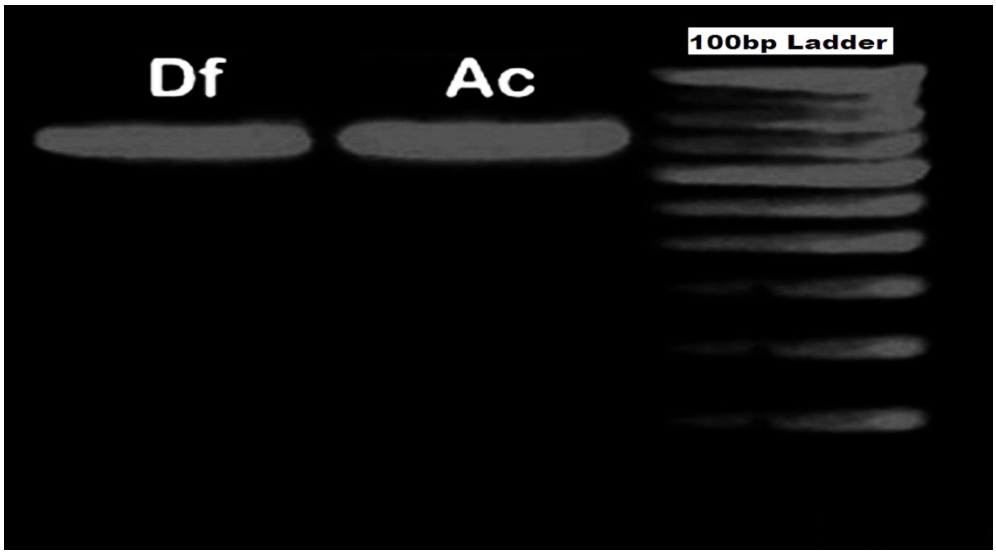

Fig. 3. Amplified serine protease gene PCR product with 100bp Ladder on 1.5% agarose gel. Ac- *Arthrobotrys conoides* and Df- *Duddingtonia flagrans*.

3.2 Molecular characterization

A 726 and 716bp gene fragment of the serine protease gene from *A. conoides* and *D. flagrans* genome respectively was amplified (Figure-3). Blastn search against NCBI database showed amplified gene sequence of *A. conoides* is 99% similarity with serine protease (AcI) reported by [22] while amplified sequence from *D. flagrans* showed 99% similarity with alkaline serine protease (PII) reported by [21]. Serine protease of our isolate of *D. flagrans* is alkaline serine protease (PII) according to its biochemical property and sequence similarity. Possible ORF was predicted at position 269-662bp for *A. conoides* and 294-639bp for *D. flagrans* serine protease gene sequence. The amino acid sequences were highly conserved with the serine protease gene from different nematophagous fungi (Figure-4). Sequences were submitted to GeneBank under accession No. KC769585 and KC862257 for serine protease gene of *A. conoides* and *D. flagrans* respectively. Phylogenetic analysis showed three different clades among serine proteases from different nematophagous fungi. Serine protease of *A. conoides* and *D. flagrans* forms a monophyletic clade with serine protease gene of other nematode trapping fungi, whereas serine proteases of egg parasitic and other nematophagous fungi were forming two separate clade (Figure-5). Our results support the conclusion of Yang et al., 2007 and Li et al., 2010 [22,32].

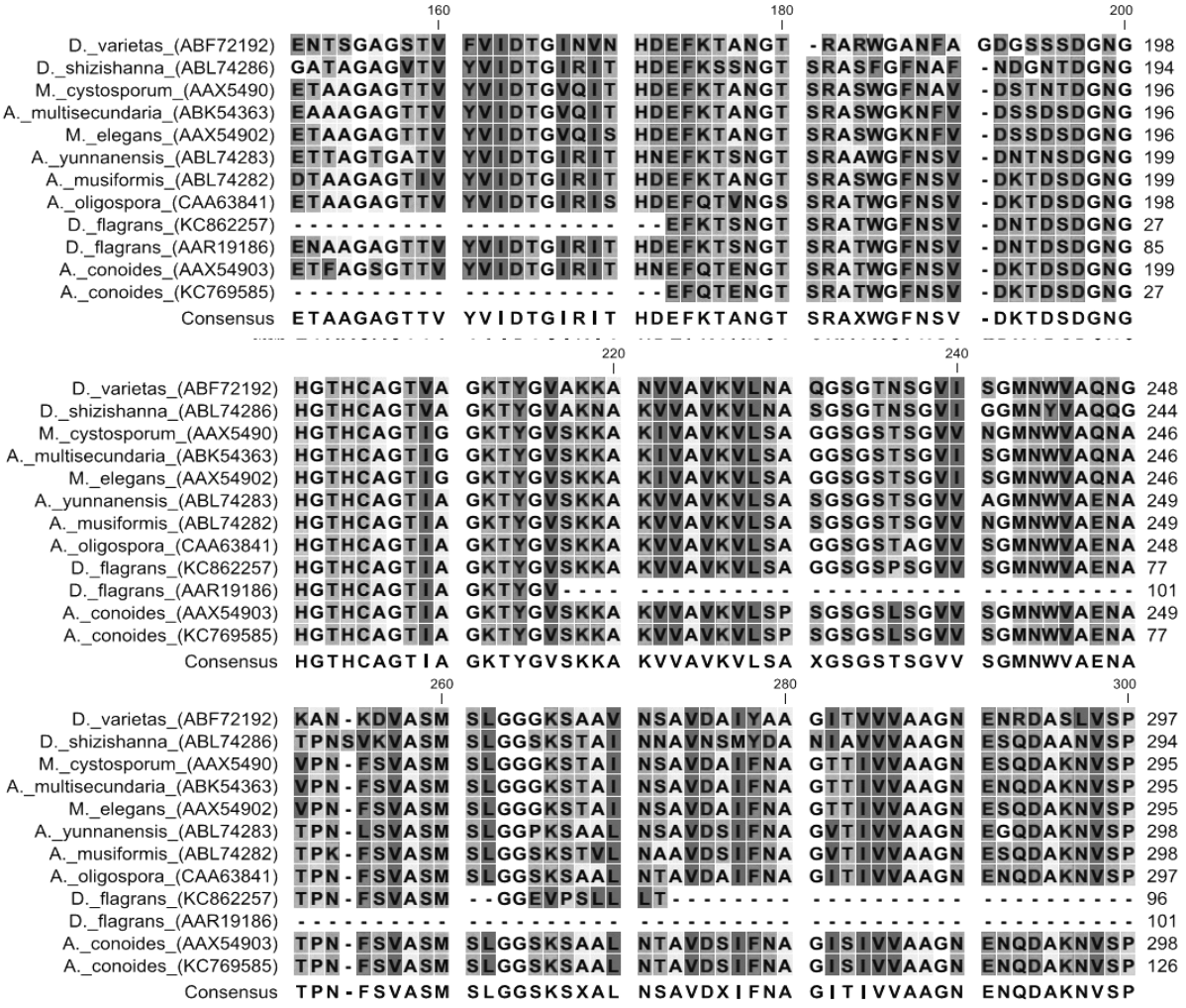

Fig. 4. Alignment of the amino acid sequence of serine protease gene ORF of *A. conoides* and *D. flagrans* with serine protease of nematode trapping fungi.

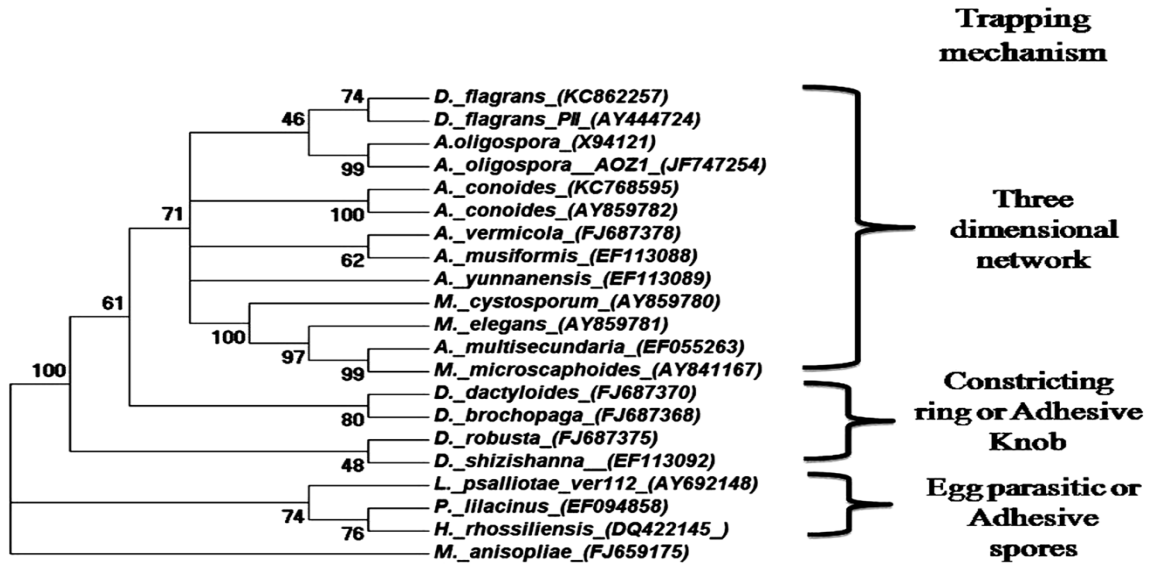

Fig. 5. The evolutionary history was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Maximum Composite Likelihood method. All positions with less than 95% site coverage were eliminated. Evolutionary analyses were conducted in MEGA5. GeneBank accessions Nos. are given in the bracket.

3.3 Induction of serine protease activity

As serine proteases have been identified as one of the major vigilance factor in nematophagous fungi [33,34], we have studied the induction in serine protease gene activity in these two fungi. Proteolytic activity was increased in the induced culture broth of both the fungi compare to control. 2.14 and 6.4 fold increases in proteases activity was observed in *A. conoides* and *D. flagrans* culture broth respectively (Figure-6). This is due to induction of both the fungi in presence of nematode for it predacious life. Further actual gene expression at molecular level was conformed through RT-PCR.

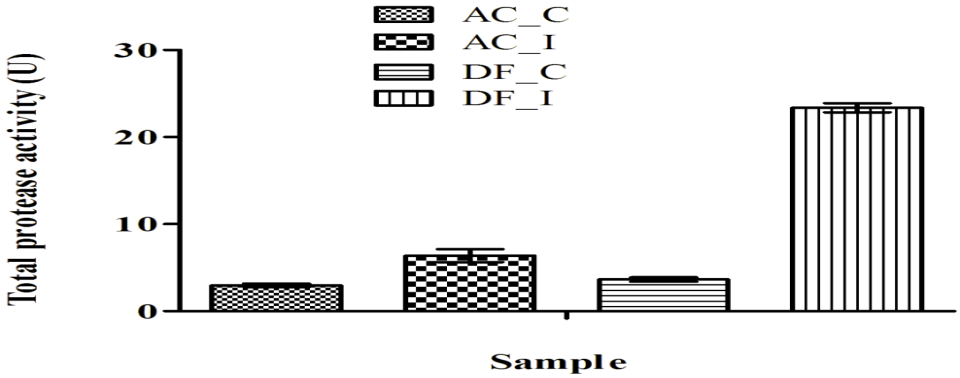

Fig. 6. Biochemical assay of proteases activity from supernatant of AC_C- *Arthrobotrys conoides* control, AC_I- *Arthrobotrys conoides* induced, DF_C- *Duddingtonia flagrans* control and DF_I- *Duddingtonia flagrans* induced sample after 24h incubation with root-knot nematodes. Bar sign on column indicates standard deviation of three replicates.

Results of RT-PCR showed massive induction in the serine protease gene in both the fungi compare to biochemical assay. This may be due to the presence of inhibitors or other chemicals which are present with crude enzyme and restrain enzyme activity during biochemical assay. Another reason behind carrying out RT-PCR assay is that absolute enzyme activity study requires multiple protein purification steps which are time consuming, tedious and loss in the enzyme concentration at each purification steps. So we have selected RT-PCR technique to quantify mRNA expression.

Real time PCR is a revolutionary technique and it is the standard method for quantifying mRNA levels from any cells. In the present study, we used RT-PCR to quantify expression of serine protease gene in *A. conoides* and *D. flagrans.* Results of present the study showed an increased level of mRNA expression of serine protease gene transcripts in both the fungi. Figure 7 shows the amplification plot of RT-PCR. 4.27 and 23.8 fold increase in *A. conoides* and *D. flagrans* serine protease gene respectively compared to the un-induced (Figure-8).

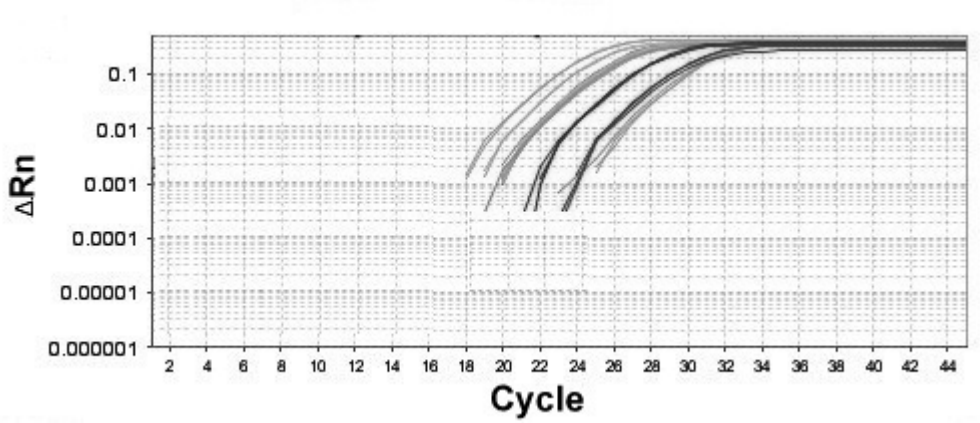

Fig. 7. Amplification plot of RT-PCR.

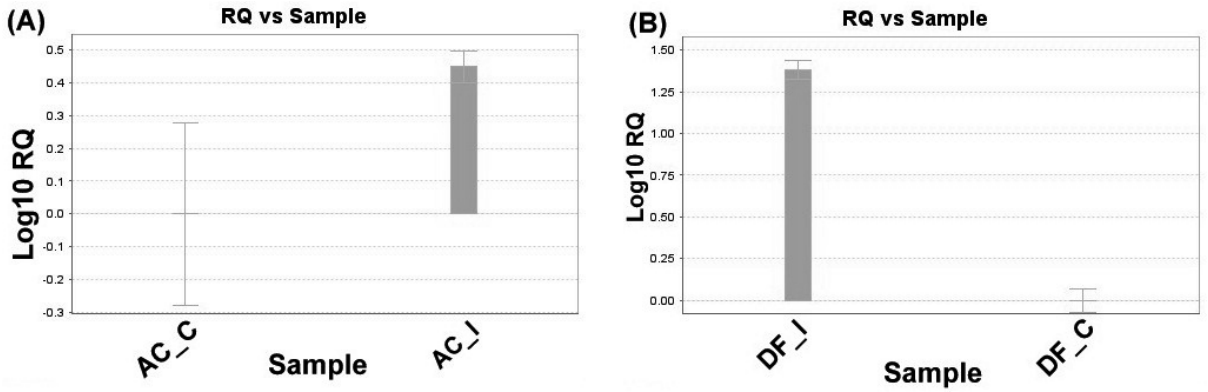

Fig. 8. Relative Quantification (RQ) graph. (A) *A. conoides* and (B) *D. flagrans.* AC_C-*Arthrobotrys conoides*, DF- *Duddingtonia flagrans.* I-Induced and C- control.

An increased level of this gene activity is probably due to physiological response of these fungi towards the nematodes; however, the level of their up regulation was different in both the fungi. Our results also support the observation of Yang and co-workers who reported up regulation of serine protease P12 and P186 by 5.9 and 23.4 fold in *A. oligospora* after 10 hours incubation with nematode extract [25] (Yang et al., 2011) and Ahren and co-workers reported up regulation of subtilicin-like serine protease (spr1) in *Monacrosporium haptotylum* during 1-24 hours during infection to *C. elegans* [24] (Ahren et al., 2005). Different types of serine proteases from nematophagous fungi have been identified and studied for pathogenicity towards nematodes and egg or cysts of nematodes [35, 36,37,38,39,40] but there is any report on induction of serine protease gene in *D. flagrans* and *A. conoides*. Up regulation of the serine protease gene in *A. conoides* and *D. flagrans* states the role of this enzyme in pathogenicity to nematodes. Serine proteases are pathogenicity related key factor not only in nematophagous fungi but also in insect pathogenic fungi, *M. anisopliae* [41]. *Beauveria bassania* [42] and *Cordyceps sinensis* [43]. This enzyme plays a key role in digestion of insect cuticle. All these studies clearly suggested that serine proteases are play a key important role in infection process in entomopathogenic and nematophagous fungi.

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