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ISOLATION, MOLECULAR CHARACTERIZATION AND PREDATORY ACTIVITY OF TWO INDIAN ISOLATES OF NEMATODE-TRAPPING FUNGI

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ABSTRACT

Two nematode trapping fungi were isolated from the arable soil samples collected from district Anand (Gujarat) India and identified on the basis of their culture-morphological characteristics and 18S rRNA gene sequencing as *Arthrobotrys conoides* (GenBank accession No. JX979095) and *Duddingtonia flagrans* (GenBank accession No. JX979094). These fungal isolates were assessed for their trapping efficiency against root knot disease causing nematode *Meloidogyne* spp., isolated from the infected tomato plants. When incubated with nematodes for 24 hr, the trapping efficiency of *A. conoides* and *D. flagrans* was 92-95 and 22-29%, respectively. Growth conditions with respect to medium, temperature and pH were also optimized. This study showed that *A. conoides* is a promising candidate to control root-knot nematodes. Phylogenetic relationship with other nematophagous fungi showed that isolates formed monophyletic clade with other nematode trapping fungi as well.

Key words: *Arthrobotrys conoides*, biocontrol, *Duddingtonia flagrans*, *Meloidogyne*, nematophagous fungi

INTRODUCTION

The increasing population of world has resulted in high demand for food. However, crop loss due to the infectious diseases appears to be more recurrent and cause great economic looses. Plant-parasitic nematodes cause a number of diseases and decrease agriculture crop production worldwide (Oka *et al*., 2000). Amongst these root-knot nematodes, *Meloidogyne* spp., infect a variety of crop plants and are considered the most devastating plant parasitic nematodes (Hajer *et al.*, 2010). Several strategies are presently employed to control plant parasitic nematodes which include sanitation, soil management, organic amendment, chemical nematicides, biological control, etc.

(Collange *et al*., 2011 Mukhtar *et al*., 2014). Among these nematicides are widely used to control nematode population due to their instantaneous results. However, the excessive use of nematicides is not only toxic to plant, soil and environment but has also resulted in the development of resistance in nematode populations (Anand *et al*., 2010). This constraint has been a major driving force behind the development of bio-products for controlling parasitic nematodes. The use of biological products to protect the plants form infectious diseases is an eco-friendly and economically feasible approach.

Soil harbours a diverse range of fungi and some of these are parasitic on nematodes (Singh *et al*., 2006). These include nematode-trapping fungi that form peculiar trapping structures by which they trap nematodes and kill them. In fact, nematophagous fungi have nowadays become a research of interest for the scientists all over the world to control parasitic nematodes. Amongst the various nematophagous fungi, many species of *Arthrobotrys* have been found to exhibit predatory activities against both plant and animal parasitic nematodes (Chandrawathani *et al*., 1998; Kumar and Singh 2006; Carvalho *et al*., 2011; Wang *et al*., 2013). On the other hand, another fungus *Duddingtonia flagrans* (Dudd.) R.C. Cook 1969 has extensively been studied for its nematophagous activity against animal parasitic nematodes (Nagee *et al*., 2001; Santurio *et al*., 2011; Buzatti *et al*., 2012). *D. flagrans* reportedly produces profuse chlamydospores which can undergo gut passage in small ruminants (Sanyal and Mukhopadhyaya 2003; Ferreira *et al*., 2011; Tavela *et al*., 2013).

Many researchers from India have reported the use of various biological agents to control root-knot nematodes (Muthulakshmi *et al*, 2010; Shankar *et al*., 2011; Sowmya *et al*., 2012). However, less attention has been paid to biocontrol agents of plant parasitic nematodes including nematophagous fungi. In India till date *Arthrobotrys oligospora*, *Dactylaria brochopaga* and *Monacrosporium eudermatum* have been reported to control *Meloidogyne* spp. (Singh *et al*., 2012a,b; Usman and Siddiqui, 2012; Saikia *et al*., 2013; Singh *et al*., 2013). The present study was aimed to assess the predatory activity of two locally isolated nematode trapping fungi against the root-knot nematode *Meloidogyne* spp. as well as to optimize the growth conditions of these two fungi.

MATERIALS AND METHODS

Isolation of nematophagous fungi and their morphological characterization

A total 23 soil samples were collected in Decembers-January, 2011 from various agricultural fields in Anand district of Gujarat. Nematodes used in the present study were collected from the infected roots of tomato plants by simply dipping chopped roots in sterilized distilled water amended with tetracycline 30 µg mL^{-1} for 30 min. and collected by centrifugation at 2000 rpm for 3 min. Nematodes were washed three times with sterilized water containing tetracycline and collected each time as mentioned above. The fungi from soil were isolated on water agar (HiMedia, India) as per the method described by Nagee *et al.* (2001). The fungi showing trapping of any nematode were subsequently purified by sub-culturing on Czapek Dox agar (CDA). The purified cultures were maintained and preserved on 1.7% corn meal agar (CMA) and incubated at 28±1ºC for 6 days. The pattern of trapping structure and morphological features of conidiospores were used to identify the fungi (Drechsler, 1937; Cook and Godfrey 1964; Dayal, 1976; Schenck *et al*., 1977; Rubner, 1996). Microscopy and micrometry of fungi was done using light microscope under magnification of 10X and 40X attached with camera (Leica, MD2500). For further morphology-based identification, the conidial size was measured after growing fungi on nine different media (Singh *et al*., 2005). The size of 10 conidia form each plate was measured and average size (length) calculated.

Molecular characterization of fungi

DNA from the isolated fungi was extracted with lysis buffer containing 0.1M tris (pH 7.1), 0.3M EDTA (pH 8.0) and 1% SDS, followed by phenol-chloroform protein precipitation. It was then precipitated with 75% ethanol and finally washed with absolute ethanol. The 18S RNA gene was amplified by using universal primer F 5'GTAACCCGTTGAACCCCATT3' and R 5'CCATCC AATCGGTAGTAGCG3' (Sigma, India). The twenty five milliliter PCR reaction mixture contained 1 µL (~50-70 ng) DNA, 1 µL each primer (10 µM), 2.5 µL dNTPs mix (2.5 mM each), 2.5 µL 10X *Taq A* assay buffer, 1.5 U *Taq* DNA polymerase and 16.5 µL MiliQ water. *Taq* DNA, dNTPs were purchased from Genei, Bangalore. Amplification of target sequence was carried out in thermocycler (Corbett, Korea) with cycling profile of pre-PCR at 94ºC for 5 min., followed by denaturation at 94ºC for 1 min., primer annealing at 58ºC for 1 min. and elongation at 72ºC for 1 min. for 40 cycles, The final extension was carried out at 72ºC and the PCR products electrophoresed in 1% agarose gel in submarine system and visualized on UV transiluminator. Amplified products were sent to Eurofins Genomics India Pvt. Ltd., Bangalore (India) for sequencing. The sequences were analyzed using nucleotide sequences and deposited in GeneBank. The phylogenetic relationship with 13 different species of nematophagous fungi having different mechanism to infect/trap nematodes was conducted by MEGA5.1 (Tamura *et al*. 2011) using Neighbor-Joining (NJ) method (Saitou and Nei, 1987).

Predatory activity against Meloidogyne spp.

Nematode trapping efficiency of isolated fungi was performed in 90 mm dia. Petri-plates containing half strength CMA. Mycelial agar discs (8 mm dia.), form 7 days old CMA plate grown at 28±1ºC, were inoculated upside down on half strength CMA and allowed to grow for 5 days. On 6th days 200 µL containing ~1000-1100 nematodes (adult and larvae) were added to each plate and incubated in dark at 28±1ºC for 24 hr. After 24 hr the plates were observed under inverted light microscope for trapping of nematodes. On 7th day the plates were flooded with 2 mL normal saline solution to recover untrapped nematodes. About 100 µL of this suspension was deposited on glass slide (6 replicates) and the number of nematodes counted under light microscope. The mean number of nematodes in these aliquots was used to find the total number of nematodes trapped by fungi. The mean number of nematodes recovered form treated groups against the mean number of nematodes recovered in control plates was used to calculate percent trapping efficiency of *D. flagrans* and A. *conoides.* The data was statistically analyzed by one way ANOVA to compare parasitism of isolates against control group.

Optimization of growth conditions

The nematophagous fungal isolates were studied for their optimum growth conditions i.e. suitable medium, pH and temperature. Nine media *viz*., corn meal agar, Czapek Dox agar, Sabouraud's dextrose agar, potato dextrose agar, Richard's agar, Jensen's agar, yeast extract peptone soluble starch agar (YEPSSAM), Martin's agar and nutrient agar were evaluated as per the method described by Singh *et al*. (2005). For optimum pH determination, the isolates were grown on CMA but the pH values were varied *viz*., 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0. For temperature studies, the isolates were allowed to grow on CMA (pH 7) and incubated at various temperatures *viz*., 4, 15, 25, 30, 37 and 42ºC. All the above studies were performed in a completely randomized design. Each treatment was replicated three times. For optimizing media and pH, the inoculated plates were incubated at 28±1ºC for 5 days. On 6th day the mean radial growth was measured and graphs plotted.

RESULTS AND DISCUSSION

Isolation and morphological characterization of nematophagous fungi

The morphology of conidia and type of trapping structure forms the basis for the identification of nematophagous fungi. Fungal isolate 'RPAN12' was found to trap the nematode by means of three dimensional net made up of 3-7 rings (Fig. 1a,d). It produced mono-septate elongated conidia measuring 19.11-31.61 µm in length (avg. length 24.27 µm) [Table 1] on erect conidiophores (Fig. 1b,c). The conidiophores are unbranched bearing upto 18 conidia. The characteristics were found similar to *Arthrobotrys conoides* Drech. (Drechsler, 1937), except for the conidial size who reported it to be 30-46 µm and the number of conidia upto 30 on each conidiophore. The size observed was slightly variable than those of Falbo *et al.* (2013). In case of another fungal isolate 'RPAN-10' ring-like structure was observed but these did not possess typical three dimensional networks like that of *A. conoides* (Fig. 2a,b). Conidia were simple, mono-septate and borne on erect conidiophores, measuring 21.38-53.37 µm length with avg. length 33.86 µm (Table 1). The conidiophores initially produced single terminal conidia but later produced consecutive conidia slightly below and to one side of older conidia (Fig. 2c). These characteristics corroborate with *Duddingtonia flagrans* (Dudd.) R.C. Cook reported by Cook (1969), except for conidial size. *D. flagrans* also produced thick walled chlamydospores (Fig. 2d). Based on these morphological characters the isolate 'RPANT10' was identified as *Duddingtonia flagrans.* Previously *Arthrobotrys oligospora*, *A. oviformis* and *D. flagrans* have been isolated and their predatory activity against animal parasitic nematodes *viz*., *Haemonchus contortus* reported by our group (Mukhopadhyaya *et al.*, 2001; Sanyal and Mukhopadhyaya 2003; Nagee *et al*., 2008). Here we report the isolation of *Arthrobotrys conoides* with nemtophagous activity for the first time from India. Our previous studies were particularly confined to the parasitism against animal parasitic nematodes, using *D. flagrans* and *A. oligospora*; however in this study successfully could target plant parasitic nematodes using *A. conoides* and *D. flagrans.*

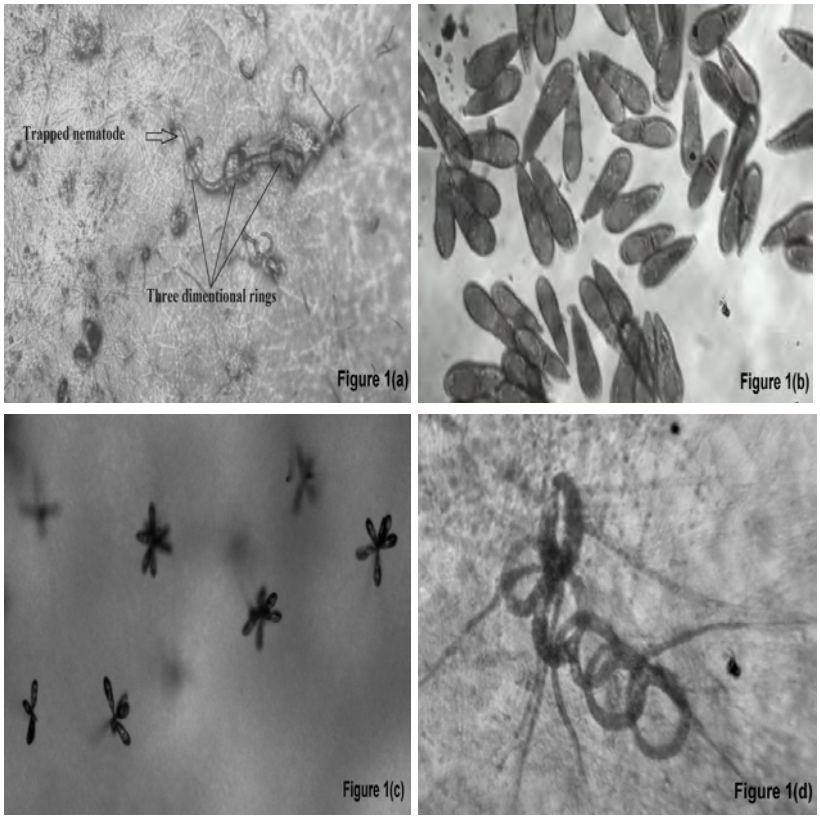

Fig. 1: ***Arthrobotrys conoides*** **(top from left to right) i. A nematode trapped in net-like structure of *A. conoides* on CMA; ii. Conidia with single septa (40X); iii. Conidia on erect conidiophore (40X) iv. (Bottom) Net-like trapping structure made up of 6 rings (40X).**

Table 1: Variation in the size of conidia of *Arthrobotrys conoides* and *Duddingtonia flagrans* on different media after incubation at 28°C for 6 days

Medium	*A. conoides*		*D. flagrans*	
	Length range (μm)	Average conidial size (μm)	Length range (μm)	Average conidial size (μm)
Corn meal agar	22.27-27.68	25.64±1.24	21.65-47.50	36.10±8.22
Czapek Dox agar	21.74-25.32	23.90±1.25	31.17-53.37	40.65±6.53
Sabouraud's dextrose agar	21.61-28.25	24.99±2.30	28.88-38.92	29.97±5.37
Potato dextrose agar	20.08-30.80	23.70±3.07	23.26-40.46	33.44±4.77
Richard's agar	19.11-26.28	23.13±2.04	21.38-38.92	29.97±5.37
Jensen's agar	23.45-25.31	23.01±1.89	23.57-42.09	31.61±5.64
YEPSSAM*	20.84-28.46	24.63±2.61	22.28-40.25	33.75±6.39
Martin's agar	21.78-31.61	25.12±2.66	21.61-30.01	26.16±2.92
Nutrient agar	20.89-27.46	23.01±1.89	28.19-44.80	36.26±6.19

* YEPSSAM = Yeast extract peptone soluble starch agar medium

Molecular characterization of fungi

The identification of nematophagous fungi using small and large ribosome coding gene sequences is considered to be more accurate and precise mean (Yang and Liu., 2006; Liu *et al*., 2009). 18S rRNA genes from both the fungi were amplified and sequenced (Fig. 3). Sequences obtained were blasted in order to identify the isolates. Blast results confirmed the isolate 'RPAN-12' to be *Arthrobotrys conoides* and isolate 'RPAN-10' to be *Duddingtonia flagrans* which also matched the morphology-based identification. The sequences were submitted to the Gene Bank under Accession No. JX979095 and JX979094 for *Arthrobotrys conoides* and *Duddingtonia flagrans*, respectively). The phylogenetic relationship of nematophagous fungi based on 18S rRNA gene, nuclear loci and elongation factor 1-alpha (EF1-a) sequences have been studied previously (Ahren *et al*., 2003; Mayer and Carta, 2005) form which it may be conclude that trapping device plays a

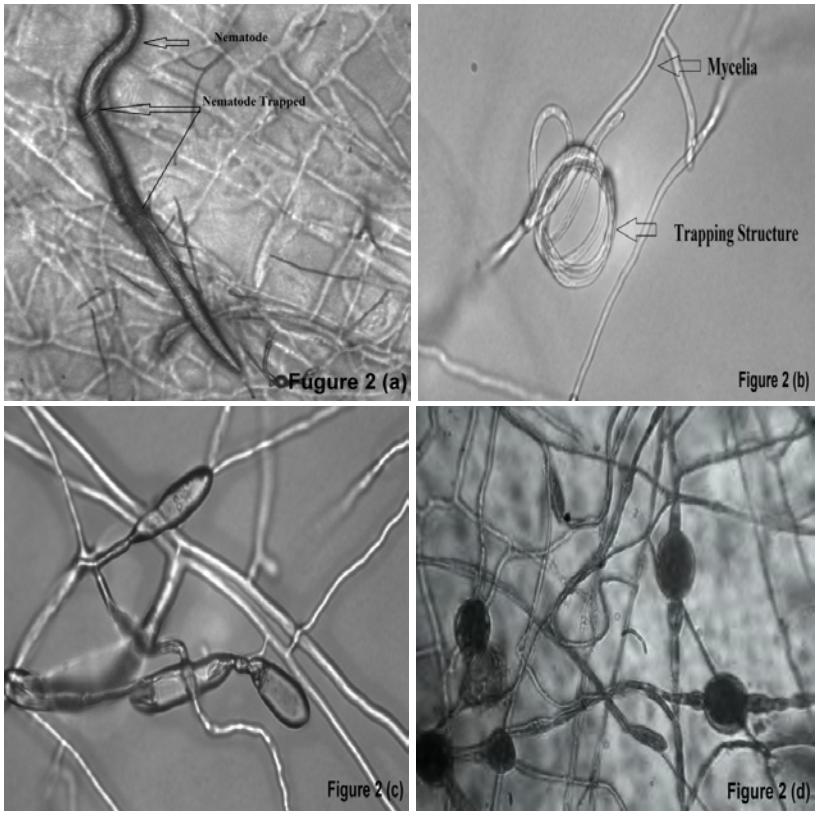

Fig. 2: ***Duddingtonia flagrans:*** **Nematode trapped in ring-like structure of *D. flagrans* (top, left); Ring-like trapping structure (40X) [top right]; Conidiospores (40X) [bottom left]; Chlamydospores (40X) [bottom right]**

very important role in classifying nematophagous fungi. These fungi have been classified into three main group i.e. nematode trapping, endoparasitic and egg/cyst parasitic fungi based upon the mechanisms they use to trap the nematodes (Rubner, 1996). All these groups form separate clades when phylogeny of nematophagous fungi is studied on the basis of 18S rDNA sequences (Ahren *et al*., 1998). In the present study, *A. conoides* and *D. flagrans* fall in the same cluster of nematode trapping fungi with bootstrap value 98 while other fungi used in phylogeny study which have different mechanism of infection form separate clade (Fig. 4).

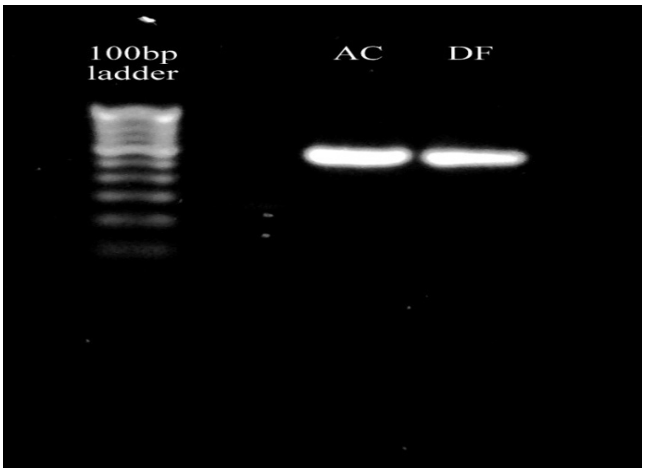

Fig. 3: 18S rRNA gene amplified PCR product with 100bp DNA marker. AC - *A. conoides* and DF - *D. flagrans*

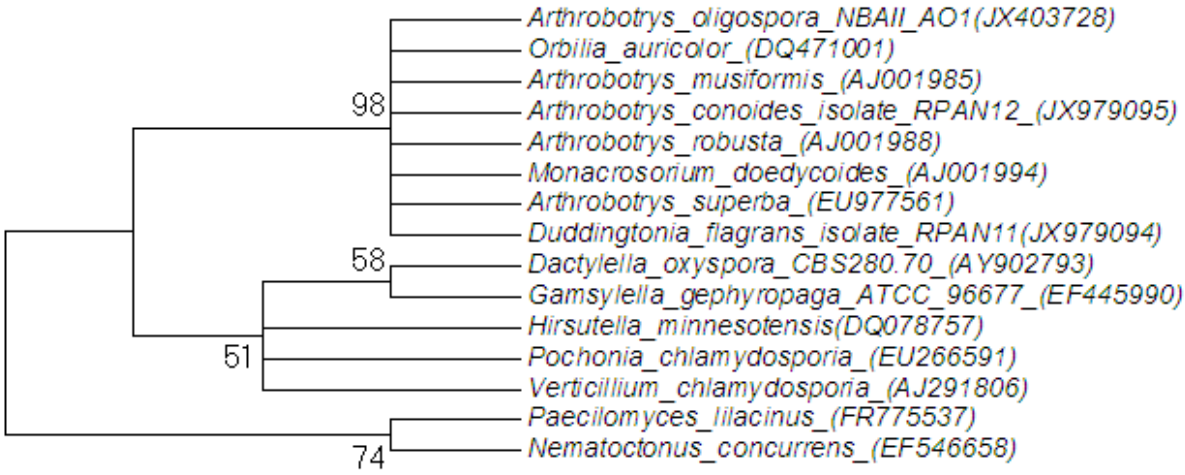

Fig. 4: Neighbor-Joining tree constructed using MEGA5. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed.

Predatory activity against Meloidogyne spp.

Very few trapping structures were produced by the isolates when grown on half strength CMA for 5 days. The numbers of trapping structures drastically increased when nematodes were added to growing fungi. Presence of nematodes induced both the isolates to form trapping weaponry. This may be due to pheromones (ascarocides) secreted by nematodes as described by Hsueh *et al*. (2013) which might trigger some signaling pathways in nematophagous fungi which ultimately lead to aggressive behaviour of these fungi. *A. conoides* started trapping nematodes after 5-6 hr incubation, and in 24 hr it showed 92-95% trapping. In comparison, *D. flagrans* showed very low trapping efficiency and only 26-29% nematodes were trapped within 24 hr. A significant difference ($P<0.001$) in trapping efficiency was observed among control, *A. conoide*s and *D. flagrans* (Fig. 5). *A. conoides* proved was more proficient in trapping of root-knot nematodes than *D. flagrans*. The difference in efficiency may be attributed to the difference in trapping structure of fungi. *A. conoides* trapped nematodes by means of adhesive network made up of 3-7 rings (Fig.1d) while *D. flagrans* trapped nematodes by ring-like structure and did not form any network characteristic like

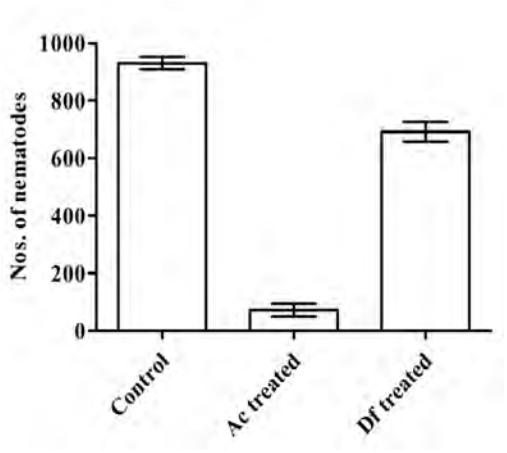

Fig. 5: Mean number of untrapped nematodes recievered from control, *A. conoides* and *D. flagrans* treated plates after 24 hr incubation. Bar represent standard deviation.

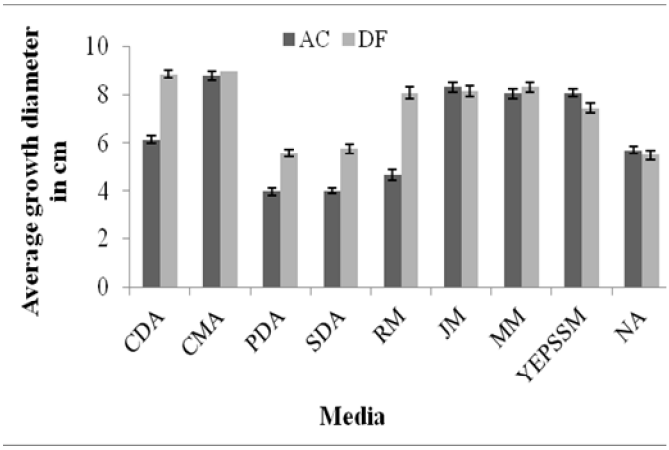

Fig. 6: Effect of media on growth of *A. conoides* (AC) and *D. flagrans* (DF): CDA = Czapek Dox agar, CMA = corn meal agar, PDA = potato dextrose agar, SDA = Sabouraud's dextrose agar, RM = Remington's medium, JM = Jensons's medium, MM = Martionson's medium, YEPSTM = yeat extract peptone soluble starch meduim and NA = nutrient agar

that of *A. conoides* (Fig. 2b). Nematophagous fungi reportedly are host-specific to some extent and have extensively been studied against animal parasitic nematodes rather than plant parasitic nematodes (Vilela *et al*., 2012; Assis *et al*., 2013). *A. conoides* has been found to be superior in controlling *Meloidogyne* spp. infection (Campos and Campos, 1997; Kalele *et al*., 2010; Falbo *et al*., 2013). Though the present study revealed the trapping efficiency of local isolates in plate assay, the appropriate formulation and application methodology for *A. conoide* needs to be devised so as to control the *Meloidogyne* spp. infection effectively.

Optimization of growth conditions

For field application mass production of fungi is required, for which appropriate cultural conditions *viz*., pH, temperature and media need to be optimized. Of the 9 media tested, corn meal agar (CMA) showed luxurious growth of both the fungi. *D. flagrans* also grow well on Czapek Dox agar (CZP), followed by Martinson's medium (MM), Jenson's medium (JM), yeast extract peptone soluble

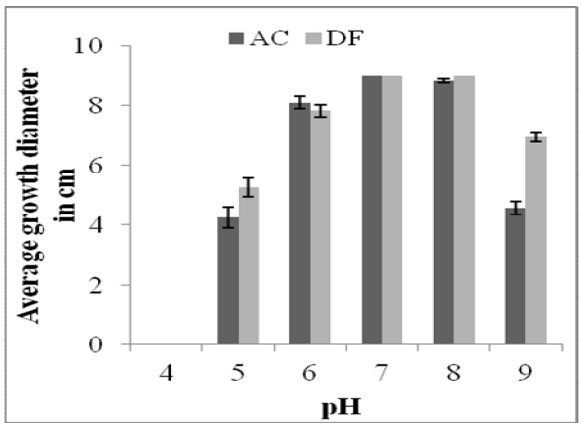

Fig. 7: Effect of pH on growth of *A. conoides* (AC) and *D. flagrans* (DF)

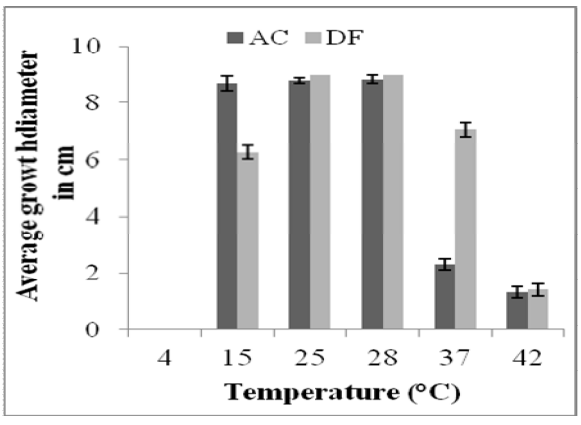

Fig. 8: Efeect of temperature on growth of nematode trapping fungi *A. conoides* (AC) and *D. flagrans* (DF).

starch medium (EXPSTM) and Remington's medium (RM). *A. conoides* showed moderate growth on RM as compared to *D. flagrans.* Both the isolates showed slow growth on Sabouraud dextrose agar (SDA), potato dextrose agar (PDA) and nutrient agar (NA) [Fig. 6]. *A. conoides* comparatively grew slowly than *D. flagrans*, whereas both the isolates showed optimal growth at 25 and 28ºC. Stunted growth of both the fungi was observed at 42ºC; while as the isolates failed to grow at 4ºC (Fig. 7). This revealed that very high and low temperatures were inhibitory for both the fungi. Healthy growth of isolates was observed in the pH range of 6 to 8 whereas at pH 5 and 9 moderate growth was noticed and no growth at pH 4 (Fig. 8). The results showed that very acidic and alkaline conditions are not conducive for the growth of both the fungi.

Conclusion: In search of potential biocontrol agent for the control of plant parasitic nematodes, two different nematode trapping fungi *A. conoides* and *D. flagrans* was isolated. *A. conoides* was found more potential candidate to control *Meloidogyne* spp. which causes root-knot. Appropriate formulation and application of *A. conoides* can serve a better prospective means to control *Meloidogyne* spp.

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