World J Microbiol Biotechnol (2017) 33:65
DOI 10.1007/s11274-017-2232-7

ORIGINAL PAPER

RNA-Seq reveals the molecular mechanism of trapping and killing of root-knot nematodes by *nematode-trapping fungi*

Ramesh Pandit[2] · Reena Patel[1] · Namrata Patel[2] · Vaibhav Bhatt[3] · Chaitanya Joshi[2] · Pawan Kumar Singh[4] · Anju Kunjadia[1]

Received: 16 October 2016 / Accepted: 17 February 2017 / Published online: 4 March 2017

Abstract Nematode-trapping fungi are well known for their inherent potential to trap and kill nematodes using specialized trapping devices. However, the molecular mechanisms underlying the trapping and subsequent processes are still unclear. Therefore, in this study, we examined differential genes expression in two nematode-trapping fungi after baiting with nematode extracts. In *Arthrobotrys conoides*, 809 transcripts associated with diverse functions such as signal transduction, morphogenesis, stress response and peroxisomal proteins, proteases, chitinases and genes involved in the host-pathogen interaction showed differential expression with fold change (>±1.5 fold) in the presence of nematode extract with FDR (p-value < 0.001). G-proteins and mitogen activated protein kinases are considered crucial for signal transduction mechanism. Results of qRT-PCR of 20 genes further validated the sequencing data. Further, variations in gene expression among *Duddingtonia flagrans* and *A. conoides* showed septicity of nematode-trapping fungi for its host. The findings illustrate the molecular mechanism of fungal parasitism in *A. conoides* which may be helpful in developing a potential biocontrol agent against parasitic nematodes.

Keywords *Arthrobotrys conoides* · *Duddingtonia flagrans* · Nematophagous fungi · qRT-PCR · RNA-Seq

Introduction

Root-knot nematodes are one of the most important pests, which causes considerable economic losses worldwide (Oka et al. 2000; Westphal 2011). For decades, several chemical pesticides are being used to control them, however, continued and excess use had led to resistance in the nematodes. Hence, use of potential biocontrol agents to combat parasitic nematodes population is one of most reliable and safe alternative. Nematophagous fungi are the predators of nematodes which can easily trap and devour them completely. Based on the infection processes, these fungi can be classified into three different groups i.e., nematode-trapping, endo-parasitic and eggs or cysts-parasitic. Nematode-trapping fungi forms trapping devices of diverse morphology to capture the nematodes which can be either adhesive network, hyphae, branches, knobs and non-constricting or constricting rings (Ahren and Tunlid 2003; Schmidt et al. 2007; Yang et al. 2007d). Among these, net forming fungi are of special interest and have been extensively studied (Braga et al. 2014; Niu and Zhang 2011; Tunlid and Ahren 2011). The unique nature of these fungi draws attention of researchers to study and use them as biocontrol agents against plant and animal parasitic nematodes.

Electronic supplementary material The online version of this article (doi:10.1007/s11274-017-2232-7) contains supplementary material, which is available to authorized users.

✉ Anju Kunjadia
anjuaribas@gmail.com

[1] Center for Interdisciplinary Studies in Science and Technology, Sardar Patel University, New V.V. Nagar, Anand 388 121, Gujarat, India

[2] Department of Animal Biotechnology, College of Veterinary Science and A.H., Anand Agricultural University, Anand 388 001, Gujarat, India

[3] Department of Pharmaceutical Sciences, Saurashtra University, Rajkot 360 005, Gujarat, India

[4] Value Addition Research and Development Department-Human Health, National Innovation Foundation (NIF)-India, Mahudi Road, Ganhdinagar 382650, Gujarat, India

Arthrobotrys conoides and *Duddingtonia flagrans* both are nematode-trapping fungi, belongs to order *Orbiliales* and family *Orbiliaceae* of *Ascomycota* and they trap the prey nematodes using their adhesive hyphal network (Drechsler 1937, 1941; Duddington 1949). *A. conoides* kills both plant as well as animal parasitic nematodes (Al-Hazmi et al. 1982; Araujo 1998; Kalele et al. 2010) and its nutrient requirements (Coscarelli and Pramer 1962; Grant 1962) and morphology (Falbo et al. 2013) is well recognized. On the other hand, *D. flagrans* controls gastrointestinal parasites of animals (Gronvold et al. 1993; Hertzberg et al. 2001), however, there are no evidences for its predacious activity against plant parasitic nematodes. In one of our previous studies, we noticed that *A. conoides* (isolate RPAN-12) efficiently traps and kills root-knot nematodes than *D. flagrans* (isolate RPAN-10) (Pandit et al. 2014b) and this created curiosity to understand the underlying molecular mechanism for this process. Till now only serine protease (AcI) of *A. conoides* is known to be involved in the infection of nematodes (Yang et al. 2007a). Additionally, recent research on *A. conoides* also explained the morphological plasticity using the electron microscopy (Falbo et al. 2013).

It is very essential to understand the molecular mechanisms of organisms in order use them as an effective biocontrol agents (Davies 2005; Morton et al. 2004). Recent advances in sequencing technology, especially next generation sequencing (NGS) has opened new horizons in this area. Genome and transcriptome analysis of numerous fungi are available, but there are limited reports for nematode-trapping fungi (Ahren et al. 2005; Andersson et al. 2014; Meerupati et al. 2013; Yang et al. 2011b). Moreover, metabolites obtained from nematophagous fungi have also been identified, which expressed nematicidal activity (Degenkolb and Vilcinskas 2016a, b). However, similar information for *A. conoides* is missing. RNA-Seq is considered as robust methodology to study the differential gene expression of an organism under different set of conditions. In the present study, we analyzed the differentially expressed genes in *A. conoides* and *D. flagrans* after inducing them with *Meloidogyne* sp. nematode mixture, with the major focus on *A. conoides*. Understanding the genes associated with the virulence are very helpful for further improvement of the strains for their predacious activities.

Materials and methods

Microorganism and culture conditions

The fungal isolates, *Arthrobotrys conoides* RPAN-12 (GenBank accession No. JX979095) and *Duddingtonia flagrans* RPAN-10 (GenBank accession No. JX979094) used in the present study were originally isolated from the soil nearby Anand Gujarat (India), identified and characterized (Pandit et al. 2014b). As reference genome of *A. conoides*, is not available, transcriptomes of both fungi were sequenced under two different conditions, i.e., in the presence and absence of nematode homogenous mixture to prepare in-house reference file for RNAseq analysis. For this, 8 mm diameter agar blocks containing mycelia of fungi (previously grown at 28 ± 1 °C for 7 days on Corn Meal Agar) were inoculated on the Petri plates (ten for each fungus) of half strength (0.85%) Corn Meal Agar (CMA) (Hi-media, India) to provide nutrient deprived conditions and incubated at 28 ± 1 °C for 4 days. *Meloidogyne* sp. of root-knot nematodes were collected from the infected tomato plants (Department of Nematology, Anand Agricultural University, Anand, Gujarat) and crude homogenised mixture was prepared in phosphate buffer saline (PBS), pH 7.0 by grinding with sterilized mortar pestle. On 5th day, 1 ml of this mixture was added to five Petri plates of each fungus (induced) and remaining five Petri plates served as control (uninduced), which were flooded with 1 ml of PBS. All the Petri plates were further incubated at 28 ± 1 °C in dark condition for 24 h. Before RNA extraction, plates were observed with the help of light microscope (Leica DM2500) using the 10× objective to evaluate the changes between induced and un-induced fungi.

Extraction of total RNA and purification of mRNA

After 24 h, mycelia were collected from each treatment, i.e. five plates of each fungi and approximately 1/3 of mycelia were preserved in RNAlater (Qiagen, UK) and stored at −80 °C which were later on used for qRT-PCR. Form rest of the mycelia total RNA was extracted using TRIzol (Invitrogen, UK) and RNeasy Mini kit (Qiagen, UK). Total RNA was treated with RNase free DNase (Qiagen, Germany) to remove any DNA traces. Before processing for mRNA extraction, quality (integrity) of RNA was assessed using Agilent 2100 Bioanalyzer (Agilent Technologies) with the help of RNA 6000 nano kit and quantity was measured using Qubit 2.0 fluorometer (Invitrogen, USA). mRNA from the total RNA was purified using the Dyna beads Oligo $(dT)_{20}$ probes (mRNA isolation kit, Roche).

Library preparation and RNA sequencing

The purified mRNA (~500 ng) was further fragmented using RNase III and size distribution of fragmented mRNA was accessed on the Agilent 2100 Bioanalyzer using DNA High Sensitivity DNA Analysis kit. After fragmentation, purified mRNA was reverse transcribed to first strand cDNA using Ion Total RNA-Seq kit v2 (Life Technologies, USA). All the four samples (control and induced of

each fungus) were systematically labelled with different Ion Xpress™ RNA 3′ barcode primers to make barcoded libraries and second strand cDNA was synthesized using Platinum PCR SuperMix, a high fidelity mix of Ion Total RNA-Seq kit v2 (Life Technologies, USA). Size distribution of the library was evaluated by High Sensitivity DNA Analysis kit on Agilent 2100 bioanalyzer. Emulsion based clonal amplification and enrichment of the template-positive Ion Sphere Particles (ISPs) was done using Ion One-Touch™ 200 template kit v2 DL on Ion OneTouch™ two system and ion one touch ES respectively (Life Technologies, USA). Sequencing was carried out on Ion Personal Genome Machine (PGM™) version 3.0 with Ion PGM™ sequencing 300 bp chemistry and Ion 316™ chip.

De novo assembly of reads and differential gene expression analysis

The raw reads obtained from sequencing run were filtered for minimum length of 40 bases and mean quality score $Q \geq 20$ using PRINSEQ 0.20.2 (Schmieder and Edwards 2011). The reads of induced samples were mapped to *Caenorhabditis elegans* whole genome and mapped reads were eliminated. To build a reference file for expression analysis, all the good quality reads of both the fungi were pooled together to ensure maximum coverage of the probable genes. Good quality reads were assembled into non-contiguous contigs using CLC genomic workbench version 4.9 (CLC Bio, Denmark), where parameters were set as minimum contig length of 200 bp and minimum 80% overlap with 90% identity among the reads. BLASTX search of all the assembled contigs were performed against non-redundant (nr) database at NCBI with minimum E-value 1E-6 using BLAST2GO (Gotz et al. 2008) and results were further utilized to prepare a reference for gene expression analysis using costumed script.

In our previous study (Pandit et al. 2014b), *A. conoides* was found superior than *D. flagrans* in catching and killing the root-knot nematodes hence, our main focus of analysis was to make out the virulence genes of *A. conoides*. Although, we had also studied differentially expressed genes in *D. flagrans*. Differentially expressed genes were identified using RNA-Seq analysis application provided by CLC genomic workbench V4.9. Expression values were calculated in terms of Reads per Kilobase per Million mapped reads (RPKM). Statistical analysis was carried out in CLC genomic workbench. Expression values (RPKM) were normalized using scaling method (Robinson and Oshlack 2010) and transformed on log2 base scale. Functional annotations of the expressed genes i.e. BLASTX, GO, enzyme code, InterProScan and KEGG pathway analysis was performed using Blast2GO, where E-value-hit-filter was set to 1E-6. GO enrichment analysis was carried out on resulting GO annotations using Fisher's exact test (p-value < 0.001) to find out the significant categories in induced fungus. Differentially expressed genes were categorised in different groups according to the sequential events that takes place during trapping and digestion of nematodes. The differential expression of genes homologous to pathogen-host interactions (PHI) database was also carried out by mapping the reads with PHI database V 4.0. For comparative orthologous analysis with other fungi, predicted proteins sequences (7225) of *A. conoides* were compared with protein sequences of other three nematode-trapping fungi with diverse morphology of trapping structures using OrthoVenn (Wang et al. 2015) and Hypergeometric test was used for GO enrichment (p-value < 0.05).

Validation of sequencing data using qRT-PCR

In order to validate the RNA-Seq data, qRT-PCR of 20 selected differentially expressed genes in *A. conoides* were used. To design the primer for target genes, the assembled contigs of each unigenes was search for blastx and the conserved sequences were subjected to Batch Primer3 v1.0 (You et al. 2008). Primers used for the qRT-PCR study are listed in supplementary table T1. RNA was re-isolated from preserved samples and approximately 1 µg of total RNA was used for cDNA synthesis using Transcriptor First Strand cDNA Synthesis Kit (Roche, USA). qRT-PCR was performed with the help of SYBR Green Real-Time PCR Kit (Life Technologies). 15 µl PCR reaction mixture contains 2 µl cDNA, 0.25 µl each of forward and reverse primer (10 µM), 7.5 µl of 2X SYBR green mix and 5 µl water. A one step thermal cycler programme used for experiments as follow: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. This was further followed by melt curve to assess the specificity of the primers. Three genes viz. G6PD, G3PDH and glycerol kinase were used as an endogenous control to normalize the data. Three replicates of sample for each primer set were taken and fold change in gene expression was calculated using $2^{-\Delta\Delta CT}$ method and results were compared with RNA-Seq data.

Results

Both fungi were baited against the nematodes crude extract for 24 h followed by observation under the microscope. *A. conoides* makes use of an adhesive network of hyphae to clutch the prey nematodes and noticeably demonstrated move from saprophytic to predacious life style after induction. After 24 h, *A. conoides* successfully developed abundant trapping structures compared with control. However, in case of *D. flagrans* the induction was poor or not at all, in terms of formation of trapping

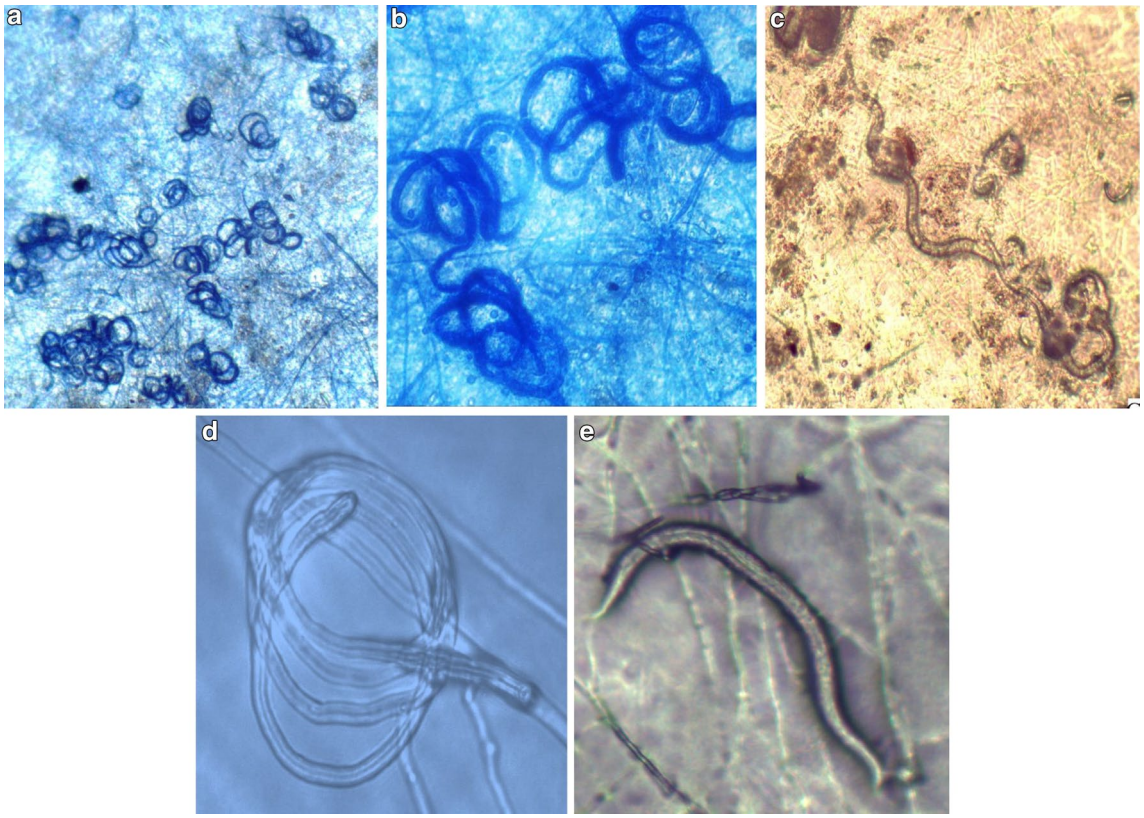


Fig. 1 Morphological characteristics of trapping structures of *A. conoides* (**a**, **b** and **c**) and *D. flagrans* (**e** and **f**) on half strength CMA. **a, b** A characteristics trapping structures of *A. conoides* under 10× and 40× respectively after induction, **c** trapped nematodes under 10× objective after 24 h incubation. **d** A characteristics trapping structures of *D. flagrans* under 40×, **e** trapped nematodes under 10× objective after 24 h incubation. (Image c and e- are from our separate experiment where we have added live nematodes on growing fungi)

devices. Figure 1 shows morphological characteristics of trapping devices of *A. conoides* and *D. flagrans*. In agreement with our previous study (Pandit et al. 2014b), the results confirm that *A. conoides* get induced when nematodes are in the vicinity.

Sequencing and de novo assembly of reads

Raw sequences obtained were submitted to NCBI sequence read archive (SRA), BioProject PRJNA238614 and PRJNA239149. Total 2.47 million reads corresponding to 415.3 million bases were retained after removing low quality reads. The filtered reads were de novo assembled to 11,602 non-contagious contigs with N50 444 bp and an average contig length of 422 bp. From these 11,602 contigs, we got hits for 9775 contigs, (9 E-value between 9.7E-06-2 to 6E–180) during BLASTx search against NCBI nr/nt database. An *in-house* reference (.gbk file) was prepared with 9775 contigs having BLASTx hit using costumed Perl script and subsequently used for RNAseq analysis.

Expression analysis and annotation

Large number of unigenes were differentially expressed (Fold change $> \pm 4$) among the control and induced fungi especially in *A. conoides* (Supplementary Fig. S1). While looking at fold change $> \pm 2$, 70 and 56 unigenes were found unique in *A. conoides* and *D. flagrans*, respectively while 77 were shared among both the fungi (Fig. 2d). While looking at PHI homologous genes, 80 and 27 unigenes were differentially expressed with fold change $> \pm 1.5$ in *A. conoides* and *D. flagrans*, respectively (Fig. 2a, b) with only four common in both (Fig. 2c, Supplementary Table T2). Hence, based on the observation of number of trapping structures, comparative genes expression and PHI homologous genes, our major focus was on gene expression in *A. conoides* for further analysis. A volcano plot clearly illustrates that large number of unigenes are differentially expressed in *A. conoides* with fold change $> \pm 1.5$ ($p < 0.01$) (Supplementary Fig. S2). Heat maps for important unigenes are shown in Fig. 3, where several unigenes with diverse functions like signal transduction, morphogenesis, hydrolytic enzymes etc. are up-regulated in *A. conoides*.

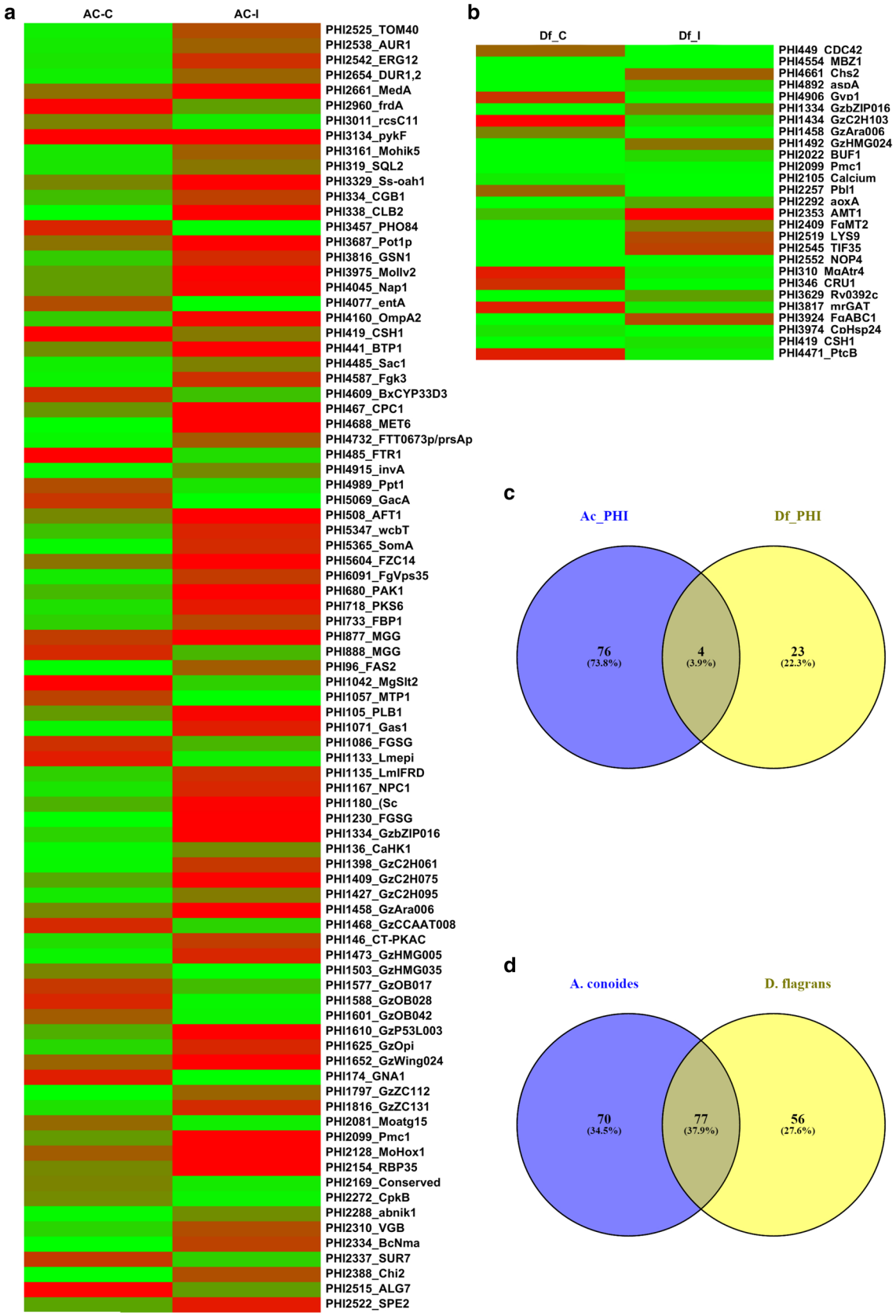


Fig. 2 Heatmap and Venn diagrams for PHI homologous genes (**a, b** and **c**) with fold change $\geq \pm 1.5$ and **d** venn diagram for gens fold change $\geq \pm 2$ in both fungi

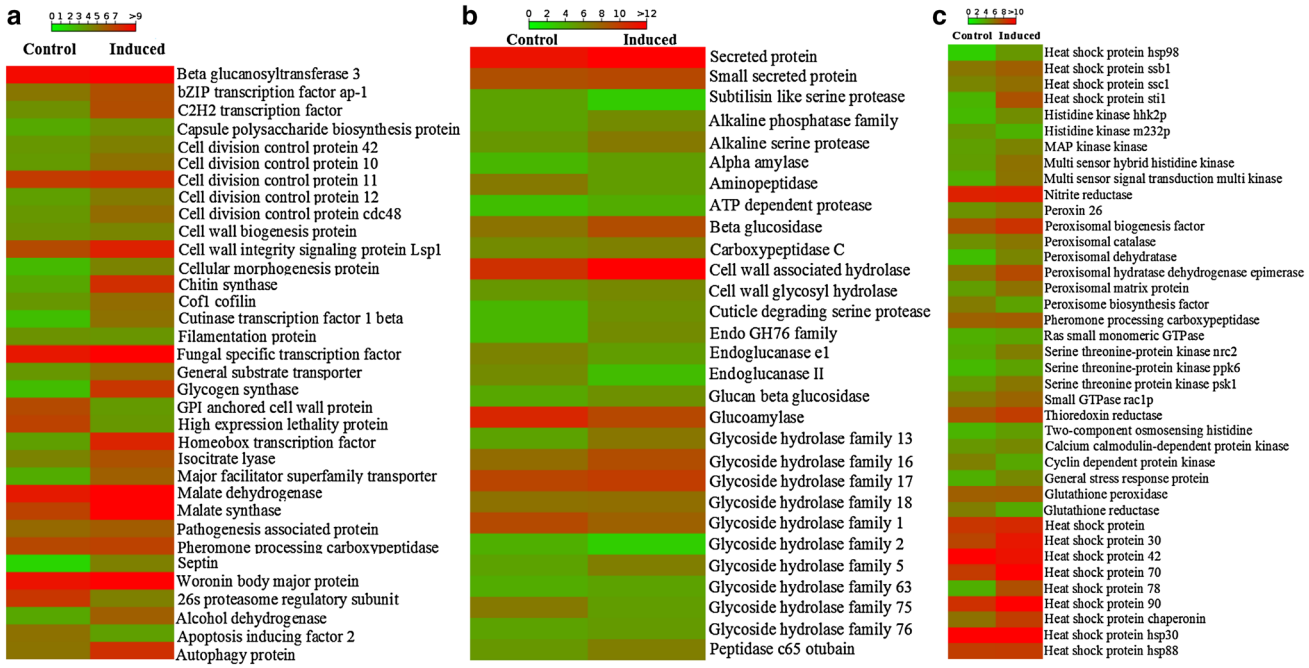


Fig. 3 Heatmap of some important differentially expressed unigenes in *A. conoides* after induction. **a** Morphogesis, cell cycle control and pathogenesis related genes, **b** hydrolytic enzymes and **c** signal transduction and stress response related genes

Considering fold change > ±2.0, 69 and 88 unigenes were up and down regulated, respectively in *A. conoides* (Supplementary Table T3). Total 1897 transcripts were uniquely expressed (i.e. having RPKM value 0) either in control or in induced fungus, out of these, 805 (Supplementary Table T4) and 1092 (Supplementary Table T5) were unique in control and induced fungus respectively. 888 unigenes were differentially expressed with fold change > ±1.5 (Log2 transformed) out of these, 809 unigenes showed significant difference ($p < 0.001$) after performing false discovery rate (FDR) test. Of the 888 unigenes, 415 unigenes were up regulated with fold change > 1.5 (Log2 transformed) and 473 are found to be down regulated with fold change > −1.5 (Log2 transformed) in induced fungus.

Further, transcripts from the control and induced samples of *A. conoides* were de novo assembled to non-contiguous contigs and annotated using Blast2GO. Best BLAST hits were obtained with E-value ranging from 1E-10 to 1E-50. Top hit species distribution graph showed the best hits with *Arthrobotrys oligospora* (a model nematode-trapping fungus), *Dactylella haptotyla* and *Drechslerella stenobrocha* (Fig. 4). GO annotation of the sequences were assigned to three basic categories, biological process (BP), molecular function (MF) and cellular component (CC). Using Fisher's exact test, enriched GO were extracted. Within category BP, tricarboxylic acid metabolic process (GO:0072350), tricarboxylic acid cycle (GO:0006099), cellular respiration (GO:0045333), citrate metabolic process (GO:0006101) and aerobic respiration (GO:0009060) were enriched in induced fungus. Among MF, genes for the nucleic acid binding (GO:0003676), nucleotide binding (GO:0000166), organic cyclic compound binding (GO:0097159), nucleoside phosphate binding (GO:1901265) and heterocyclic compound binding (GO:1901363) were abundant in induced fungus compare to un-induced one (Fig. 5, Supplementary Table T6). While in CC, no significant category was enriched in induced fungus with p-value < 0.001, however, cellular bud (GO:0005933), respiratory chain complex (GO:0098803), 1,3-beta-D-glucan synthase complex (GO:0000148), nuclear chromatin (GO:0000790) etc. were enriched with p-value < 0.05. These genes might be involved in the development of trapping structures and other morphological changes. On the other hand, in control fungus, only cytoplasmic part (GO:0044444), cytosol (GO:0005829), cytoplasm (GO:0005737), carbon–carbon lyase activity (GO:0016830) and cellular metabolic compound salvage (GO:0043094) categories were enriched with p-value < 0.001 (Fig. 5, Supplementary Table T6). Enzyme abundance was also different, oxidoreductaes and hydrolases group of enzymes were abundant in induced fungus while there was paucity of lyases. No variations were observed with isomerases and ligases class (Supplementary Fig S3). Among the top 15 KEGG pathways, enzymes associated with the TCA cycle, glycolysis, oxidative phosphorylation, methane, nitrogen, pyruvate, sucrose and starch metabolism were abundant in induced fungus,

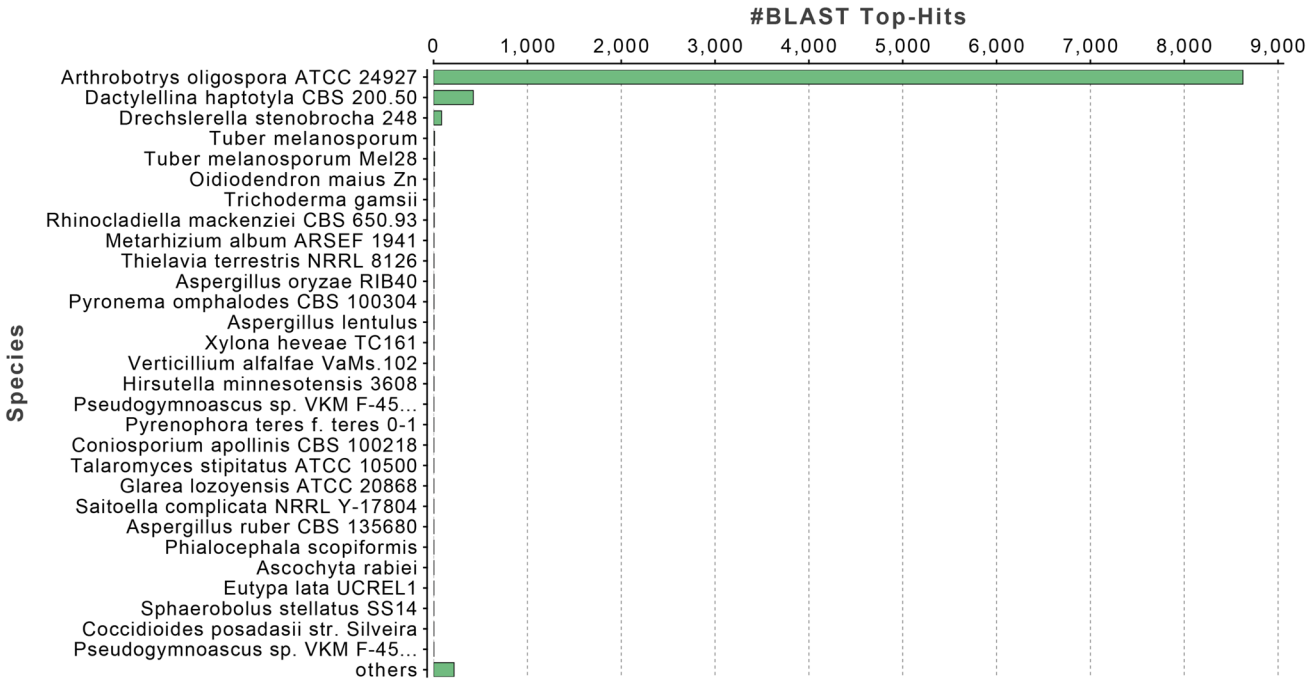


Fig. 4 Top-hit species graph from BLASTX. BLASTx was performed against NCBI nr database with minimum E-value 1E-6 using Blast2GO

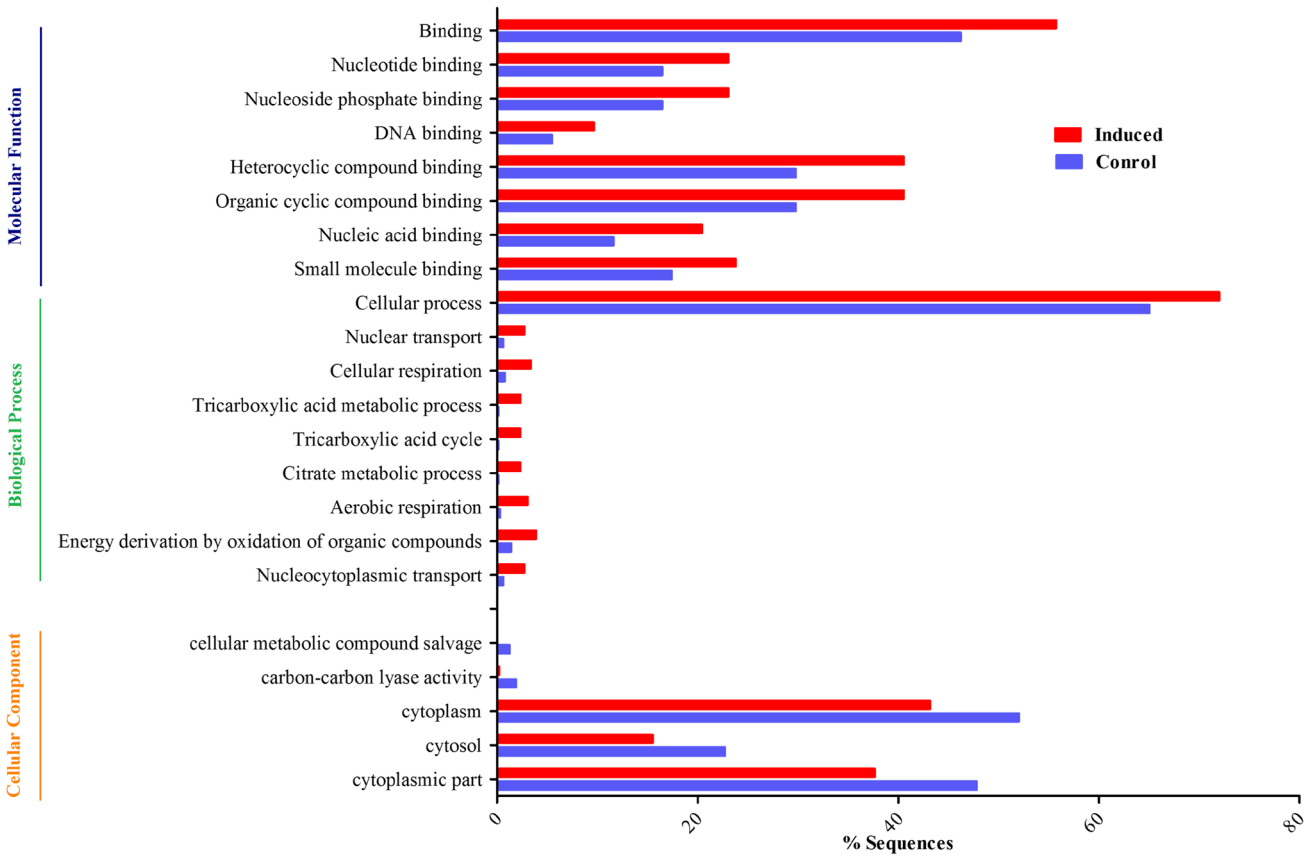


Fig. 5 Enriched gene ontology in induced and control using Fisher's exact test with p-value < 0.001. The graph was generated using Blast2GO

while as the enzymes associated with purine and pyrimidine metabolism were abundant in control fungus (Supplementary Fig S4).

We found 1152 orthologous gene clusters among all the four nematode-trapping fungi. 87 clusters were found unique for *A. conoides* (Fig. 6). Hypermetric enrichment extracted fungus specific GOs where highest, 11 GOs were enriched in *A. conoides*, majority of which were amino acid transporters and fungal-type cell wall beta-glucan metabolic process (Table 1). While comparing the homologous gene clusters, 101 and 43 were found unique in control and induced library of *A. conoides*, respectively.

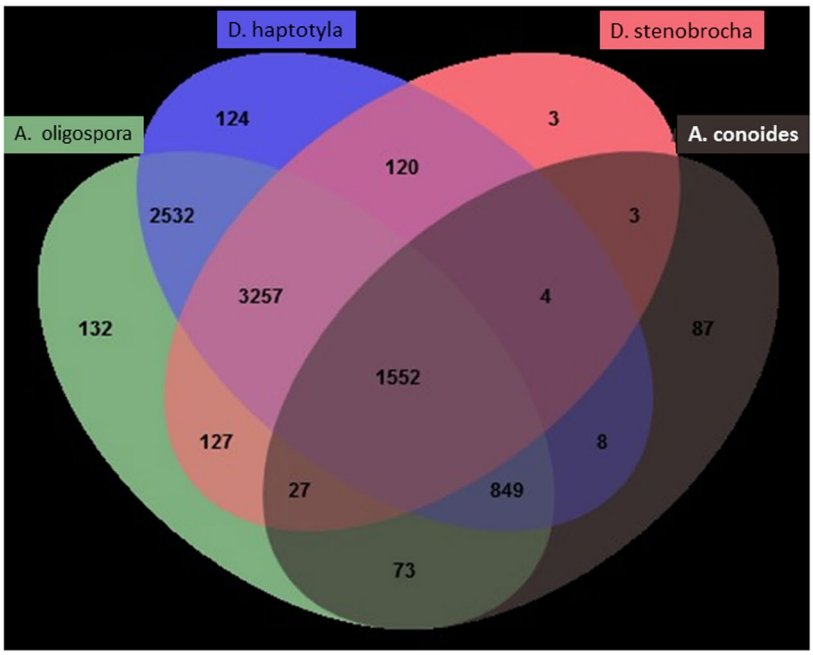
Validation of RNA-seq data using qRT-PCR

Among the three reference (endogenous control) genes, G6PD was found to be steadily expressed in both control and induced fungus, hence it was used for normalization. Of the 20 selected differentially expressed genes, majority of the unigenes showed similar profile for both qRT-PCR and RNAseq data (Fig. 7, Supplementary Table T7). Few genes showed different pattern compared to the RNA-Seq data, which may be due to technology variation (RNA-Seq vs qRT-PCR) and not using the same RNA for RNA-Seq and qRT-PCR.

Various genes involved in the morphogenesis, cell division (Fig. 2a; Table 2), hydrolytic enzymes (Fig. 2b; Table 2), signalling pathways, stress response (Fig. 2c., Table 2), energy production and conservation (Supplementary Table T8), were found up regulated in an induced sample of *A. conoides*. A proposed model for signalling in *A. conoides* is shown in Fig. 8 and proposed model of catching and killing of nematodes by *A. conoides* is demonstrated in Fig. 9. This model is somewhat similar to the previously described model for *A. oligospora* (Yang et al. 2011b) and general model described by (Li et al. 2015). In addition to all genes described, 30 hypothetical proteins were also found to be differentially expressed with fold change ±2.0 (Supplementary Table T9).

Discussion

Nematode-trapping fungi are of scientific interest as it can serve as bio-control agents for nematode pest in the agriculture. *A. conoides* is a nematode-trapping fungi which successfully trap nematodes by means of sticky ring network made up of mycelia (Saxena et al. 1987). It usually survives as a saprophyte in the soil, however, when nematodes are nearby, it transforms into predacious fungus and have a nutritious diet. As we have seen in this study as well noticed in our previous studies, presence of prey makes morphological progressions in *A. conoides*. Therefore, it is very essential to understand the molecular processes underlying this shift, in order to successfully employ it in a biocontrol programme. RNASeq is widely used method to study gene expression in various forms of life. In the present study, efforts are made to recognize the fundamental genes and their possible role in *A. conoides* during infection in the nematodes.



Species	Proteins	Clusters	Singletons
A. oligospora	11479	8549	2472
D. haptotyla	10959	8446	2097
D. stenobrocha	5597	5093	440
A. conoides	7246	2603	4253

Fungi	Morphology of trapping structure	Image of source
A. oligospora	Adhesive network	Yang et al., 2011
D. haptotyla	Adhesive Knob	Ahren et al., 2005
D. stenobrocha	Constricting ring	Liu et al., 2014
A. conoides	Adhesive network	In this study

Fig. 6 Orthologous gene clusters in *A. conoides* and other three nematode-trapping fungi

Table 1 Hypergeometric GO enrichment analysis of orthologous genes in for nematode-trapping fungi

GO ID	Function	Category	*p*-value
A. conoides			
GO:0070879	Fungal-type cell wall beta-glucan metabolic process	BP	0.007
GO:0032974	Amino acid transmembrane export from vacuole	BP	0.015
GO:0006868	Glutamine transport	BP	0.022
GO:0015828	Tyrosine transport	BP	0.022
GO:0005302	L-tyrosine transmembrane transporter activity	MF	0.022
GO:0015188	L-isoleucine transmembrane transporter activity	MF	0.022
GO:0015186	L-glutamine transmembrane transporter activity	MF	0.022
GO:0015804	Neutral amino acid transport	BP	0.022
GO:0015803	Branched-chain amino acid transport	BP	0.022
GO:1902475	L-alpha-amino acid transmembrane transport	BP	0.044
GO:0015807	L-amino acid transport	BP	0.044
A. oligospora			
GO:0019706	Protein-cysteine S-palmitoyltransferase activity	MF	0.012
GO:0008776	Acetate kinase activity	MF	0.028
GO:0006082	Organic acid metabolic process	BP	0.028
GO:0004651	Polynucleotide 5′-phosphatase activity	MF	0.028
GO:0043458	Ethanol biosynthetic process involved in glucose fermentation to ethanol	BP	0.028
GO:0004043	L-aminoadipate-semialdehyde dehydrogenase activity	MF	0.028
GO:0047735	Cellobiose dehydrogenase (acceptor) activity	MF	0.028
D. haptotyla			
GO:0036349	Galactose-specific flocculation	BP	0.005
GO:0050832	Defense response to fungus	BP	0.028
GO:0031640	Killing of cells of other organism	BP	0.028
D. haptotyla			
GO:0047145	Demethylsterigmatocystin 6-O-methyltransferase activity	MF	0.028
GO:0004503	Monophenol monooxygenase activity	MF	0.031
GO:0047145	Demethylsterigmatocystin 6-O-methyltransferase activity	MF	0.028
GO:0042438	Melanin biosynthetic process	BP	0.037
D. stenobrocha			
GO:0000298	Endopolyphosphatase activity	MF	0.002
GO:0005773	Vacuole	CC	0.028

Compared to control, abundant trapping structures were produced in *A. conoides*, after 24 h of incubation along with the nematodes extract. Though we noticed very few trapping structures on the control plates as well, however, numbers were significantly quite less and they were not much developed as observed after induction. Whereas, with *D. flagrans* no major changes were observed in the mycelia morphology on the plates. This clearly elucidate the host specificity of nematode-trapping fungi and it was in the agreement with (Andersson et al. 2014). The induction in *A. conoides* may be in response to the pheromones present in the nematodes extract (Hsueh et al. 2013). A smaller amount in fold change (<1.5 fold) with some unigenes could be explained by the induction time (24 h). This was previously observed by (Liang et al. 2013) while studying the cell wall related proteins in *A. oligospora*. Still in *A. conoides*, most of the significant genes showed up regulation even after 24 h. Many of the up-regulated unigenes viz. MAP kinases, autophagy proteins and hydrolases are similar to pathogenicity associated genes often found in *A. oligospora, M. haptotylum, M. lysipagum, P. chlamydosporia and D. stenobrocha* (Ahren et al. 2005; Andersson et al. 2013; Khan et al. 2008; Larriba et al. 2014; Liu et al. 2014; Meerupati et al. 2013; Yang et al. 2011b). Moreover, we also proposed a signalling pathway in *A. conoides* as well a model for the entire mechanism of catching and killing of nematodes. The general model for entire process is somewhat similar to the reports of (Yang et al. 2011b). This study elucidates involvement of diverse genes of nematophagous fungi during the predation and infection to nematodes. Top BLASTX hits are homologous to *A. oligospora* and fungal plant pathogens. As *A. conoides* is

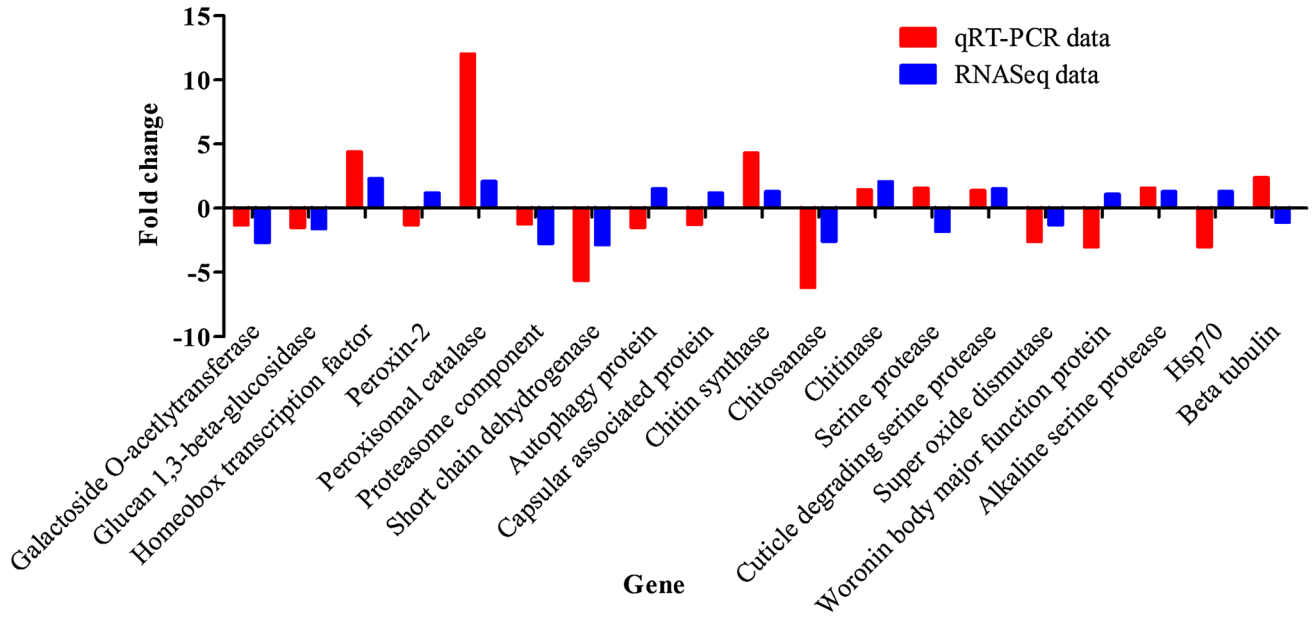


Fig. 7 Comparison of RNA-Seq versus qRT-PCR data. The graph showed the comparative fold change in *RNA-Seq* and *qRT-PCR*

a predator or in other sense pathogenic to root-knot nematodes such genes are very crucial during the predacious phase.

The de novo assembled 11,602 contigs from both the fungus libraries, which were used as reference for RNA-Seq, is very close to number of predicated genes reported in other nematode-trapping fungi *A. oligospora* (11,479) (Yang et al. 2011b) and *Monacrosporium haptotylum* (10,959) (Meerupati et al. 2013). This shows that reference which was used in expression analysis is acceptable to explain all the genes. The differentially expressed GOs in the category of BP and MF described the genes involves in different cellular processes, required for rapid cell growth and energy metabolism. Whereas in case of CC, enriched GOs are associated with morphogenesis, differentiation and development during the induction. This response might be due to the pheromones (ascarocides) (Hsueh et al. 2013) or volatile organic compounds (Li et al. 2015) and thus stimulated the fungus to produce a large number of trapping structures which eventually help to catch the nematodes. Once nematodes are trapped inside the trapping networks, *A. conoides* secrets extracellular enzymes to invade the host cuticle and simultaneously take up nutrients from the host which is evident from presence of high numbers of hydrolases during the induction. Of the unigenes with >twofold change, majority of them are hypothetical proteins and genes contributing in morphogenesis, signalling and hydrolytic enzymes. Enrichment of catalytic activity is owing to the fact that the fungus has to metabolize proteinous and other molecules accumulated from the host, which is only possible with activities of different enzymes. Similar is the case for the hydrolases and oxidoreductase group of enzymes, which are involved in the degradation of macromolecules and stress response respectively. Expression of PHI homologous genes farther aid up during the entire process. Enrichment of amino acid transport related orthologous genes in *A. conoides* explains that this fungus rapidly metabolize nematode constituents especially proteins.

According to sequential events which take place in nematode-trapping fungi, the differentially expressed unigenes are characterized into five different categories and their participation at each particular stage is discuss below.

Signaling process in *A. conoides*

Initially 'Nemin', a peptide secreted by nematodes was thought as an external signalling molecule (Pramer and Stoll 1959). Recently, it has been identified that nematode pheromones (Ascarocides) triggers trap formation in *A. oligospora* (Hsueh et al. 2013). It is widely known that a signal transduction cascade regulates fungal development and its pathogenicity (Lengeler et al. 2000). The results of our study revealed that G-proteins plus MAPKs play a very vital role in *A. conoides*, specially in sensing the presence of its prey and in other related signaling events. Proposed model for signaling (Fig. 5) where G-protein coupled receptor, G-protein subunits (α, β & γ), mitogen activated protein kinase (MAPK) mfa1, mitogen activated protein kinase kinase (MAPKK), MAP kinase kinase kinase, serine threonine protein kinases srk1, psk1 and chk2 were up-regulated during induction. Moreover, MAPK pathways assimilate

Table 2 Differential expression of some important genes in *A. conoides* after induction with nematode extract for 24 h

Unigene	Fold change	GO ID	EC
Signal transduction			
Signal transduction protein	Unique in induced	GO:0016021	
Mitogen-activated protein kinase maf1	1.2	GO:0004707	2.7.12.2
Cell wall integrity signaling protein lsp1	1.2	GO:0009408	
Histidine kinase hhk2p	2.3	GO:0007165	2.7.13.3
MAP kinase kinase	1.3	GO:0004708	2.7.12.2
MAP kinase kinase kinase	1.9	GO:0008349	2.7.11.25
Serine threonine-protein kinase nrc-2	1.48	GO:0004674	2.7.11.1
Serine threonine-protein kinase chk2	Unique in induced	GO:0004674	2.7.11.1
Serine threonine-protein kinase psk1	1.3	GO:0004697	2.7.11.1
Multi-sensor hybrid histidine kinase	1.4	GO:0000160	2.7.13.3
Histidine kinase m232p	−1.3	GO:0051704	2.7.13.3
Serine threonine-protein phosphatase 5	Unique in control	GO:0004722	3.1.3.16
Serine threonine-protein phosphatase 2a activator 1	−1.2	GO:0019211	
Serine threonine-protein phosphatase pp2a catalytic subunit	Unique in induced	GO:0004721	3.1.3.16
Serine threonine-protein phosphatase 2b catalytic subunit	Unique in induced	GO:0004723	3.1.3.16
Methyl-accepting chemotaxis protein	Unique in induced	GO:0007165	
Multi-sensor signal transduction multi-kinase	1.7	GO:0000160	
Serine threonine protein phosphatase	1.5	GO:0004722	3.1.3.16
Genes involved in morphogenesis			
Septin	3	GO:0032160	
Chitin synthase	2.9	GO:0016758	2.4.1.16
Chitin synthase activator	1.9		
Chitin synthase_d	1.4	GO:0016758	2.4.1.16
Cell wall anchored protein	1.5		
Cellular morphogenesis protein	1.7	GO:0048858	
Capsular associated protein	1.2	GO:0016740	
Capsule polysaccharide biosynthesis protein	1.3	GO:0045227	
Autophagy protein	1.5	GO:0006914	
Autophagy regulatory protein	1.1	GO:0030242	
Adenylyl cyclase-associated protein	−1.4	GO:0007010	
Chitin synthase 4	1.1	GO:0004100	2.4.1.16
Chitin synthase C	1.2	GO:0004100	2.4.1.16
Chitin synthase g	1.3	GO:0004100	2.4.1.16
Cell wall biogenesis protein	1.1	GO:0042546	
Class v chitin synthase	Unique in induced	GO:0004100	2.4.1.16
Glucan synthase	Unique in induced	GO:0003843	2.4.1.34
Peroxisomal proteins that are found in dense bodies of trapping devices			
Malate dehydrogenase	1.2	GO:0030060	1.1.1.37
Isocitrate lyase	1.3	GO:0004451	4.1.3.1
Malate synthase	1.3	GO:0004474	2.3.3.9
Peroxisome biosynthesis protein (+)	−1.4	GO:0008270	
Peroxisomal dehydratase	1.8	GO:0016702	1.1.1.35
Peroxisomal catalase	2.1	GO:0004130	1.11.1.6
Peroxisomal membrane protein	1.2	GO:0016021	
Peroxisomal matrix protein	1.4	GO:0055114	
Peroxisomal hydratase-dehydrogenase-epimerase	1.3	GO:0009405	2.4.1.119 & 1.1.1.n12
Woronin body major protein	1.1	GO:0005777	

Table 2 (continued)

Unigene	Fold change	GO ID	EC
PEX19 family protein	1.1	GO:0005777	
Peroxisomal targeting signal-1 receptor	1.1	GO:0016740	
Peroxisome biosynthesis protein (pas1 peroxin-1)	Unique in induced	GO:0005778	
Hydrolytic enzymes required to penetrate and degrade the host cuticle			
Subtilisin-like serine protease	−1.8	GO:0004252	3.4.21.62
Alkaline serine protease	1.3	GO:0006508	3.4.21
Cuticle degrading serine protease	1.5		3.4.21
Small secreted proteins	1		
ATP-dependent protease la protein	1.1	GO:0004176	3.4.21.53
Class iii chitinase 1	2	GO:0004568	3.2.1.140
Cell wall-associated hydrolase (Protease)	1.7	GO:0016787	3.4.21
Adam protease adm-b	1.1	GO:0004222	3.4.25
Zinc metalloproteinase	1.2	GO:0004190	3.4.24
Cutinase transcription factor 1 beta	2.1	GO:0000981	
26s protease regulatory subunit s10b	Unique in control	GO:0000502	
Serine protease inhibitor	Unique in control	GO:0006508	
26s protease regulatory subunit 7	Unique in control	GO:0000502	
Stress responsive genes			
Thioredoxin reductase	1.2	GO:0019430	1.8.1.9
Glutathione s-transferase	−1.9	GO:0004364	2.5.1.18
ATP-dependent chaperone	−1.1	GO:0006950	
Chaperone protein dnaj	1.3	GO:0031072	
Hsc70 cochaperone	1.2	GO:0005515	
Hsp70 chaperone	1.3	GO:0006950	
Molecular chaperone	−1.4	GO:0006950	
Nitric oxide reductase	Unique to induced	GO:0016966	1.7.3
Superoxide dismutase	−1.3	GO:0004784	1.15.1.1
General stress response protein	1.4	GO:0009408	
Stress response rci	−1.3	GO:0016021	
Response regulator 9	Unique in induced	GO:0000156	
Bifunctional aconitate hydratase 2 2-methylisocitrate dehydratase	1.6	GO:0052632	4.2.1.3
Multi-sensor hybrid histidine kinase	1.4	GO:0000160	2.7.13.3
hsp70 protein	1.4	GO:0006950	
30 kDa heat shock protein	1.1	GO:0055114	
70kdal heat shock partial	1.6	GO:0006950	
Heat shock protein 78	2.2	GO:0006950	
Heat shock protein 90	1.3	GO:0006950	
Heat shock protein 42	−1.2	GO:0006950	
Heat shock protein chaperonin	1.3	GO:0006950	
Heat shock protein hsp98	2.1	GO:0006950	
Heat shock protein sti1	2.3		
Heat shock protein ssc1	1.2		

Fold change is based on Log2 transformed expression values. The genes are categorised according their involvement in function

the signals from multiple receptor pathways including two-component signalling systems (Gustin et al. 1998). Histidine kinases are the part of basic two component system by which some organisms sense and respond to the environment (Catlett et al. 2003). In *A. conoides*, histidine kinase hhk2p and histidine kinase m232p were up-regulated in the presence of nematodes extract. This suggests that MAPKs and histidine kinases are vital for signalling in *A. conoides*. Our results also correlates with the signal transduction pathways in *A. oligospora*

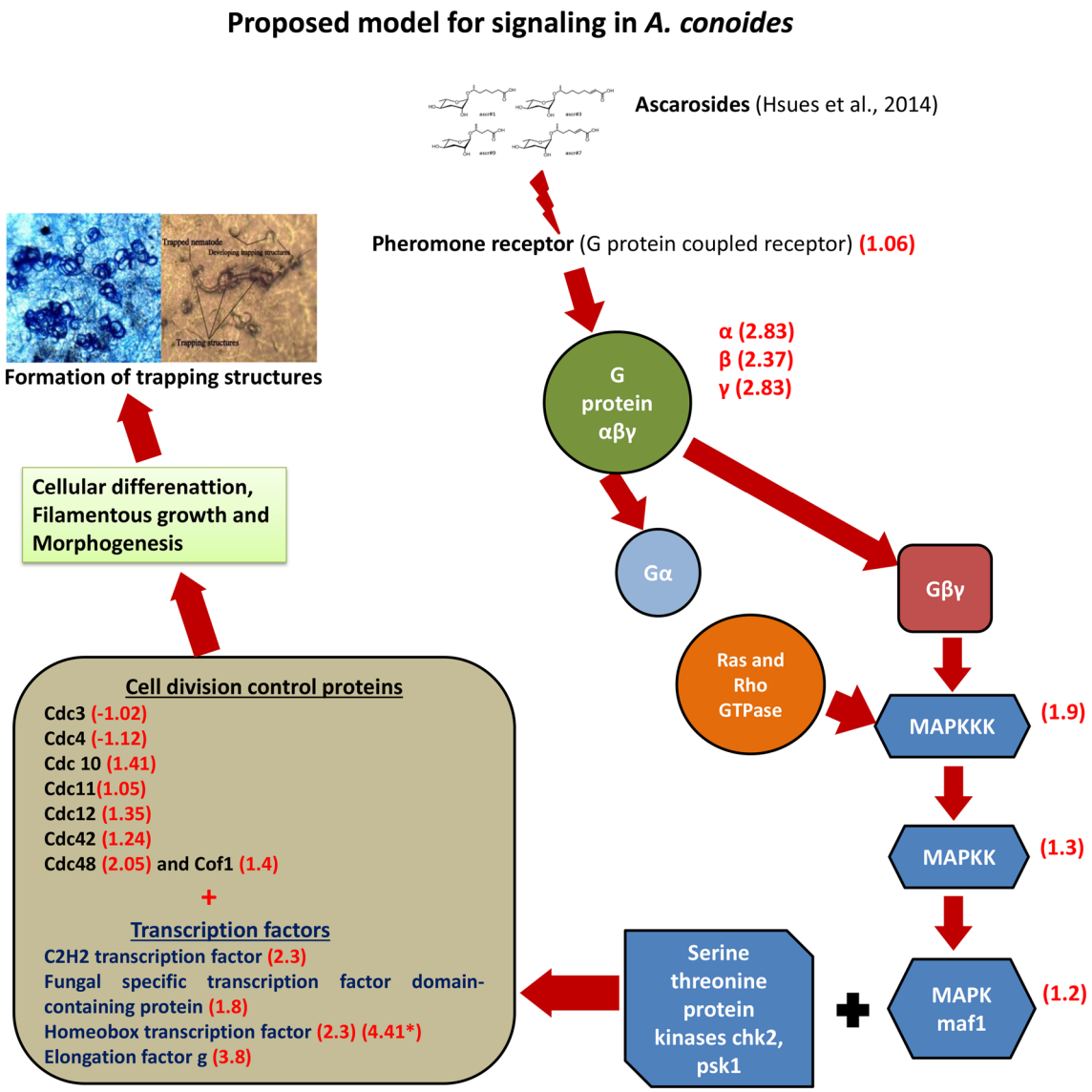


Fig. 8 A proposed model of signalling in the *A. conoides*. Expression value of each unigenes in given *red* in color in bracket. *Indicate fold change in qRT-PCR. (Color figure online)

which gets up regulated after 10 h of incubation with nematode extract (Yang et al. 2011b) and pathogenicity related genes in *M. haptotylum* (Meerupati et al. 2013), however, in both these fungi the role of G-proteins does not exists in signalling. The role of G-protein signaling is described in *D. stenobrocha* when the nematode enters into constricting ring (Meerupati et al. 2013). Thus, various differentially expressed genes of signal transduction pathways in *A. conoides* have proposed function in sensing the nematodes and for switching to predacious life form, which additionally add up in stress response later on.

Genes involved in morphogenesis and cytoskeleton building, essential to build up trapping structures

Next crucial step for infection is formation of hyphal trapping devices, which help fungus to trap the prey nematodes. *A. conoides* undergoes different cellular and morphological alterations during the process of trapping and digesting the prey nematodes. One of the key features is the formation of trapping devices, which require proteins for cell division, cell wall biogenesis, morphogenesis etc. Several key genes for morphogenesis were found to be up regulated in *A. conoides* in the presence of nematode extract. Enzymes

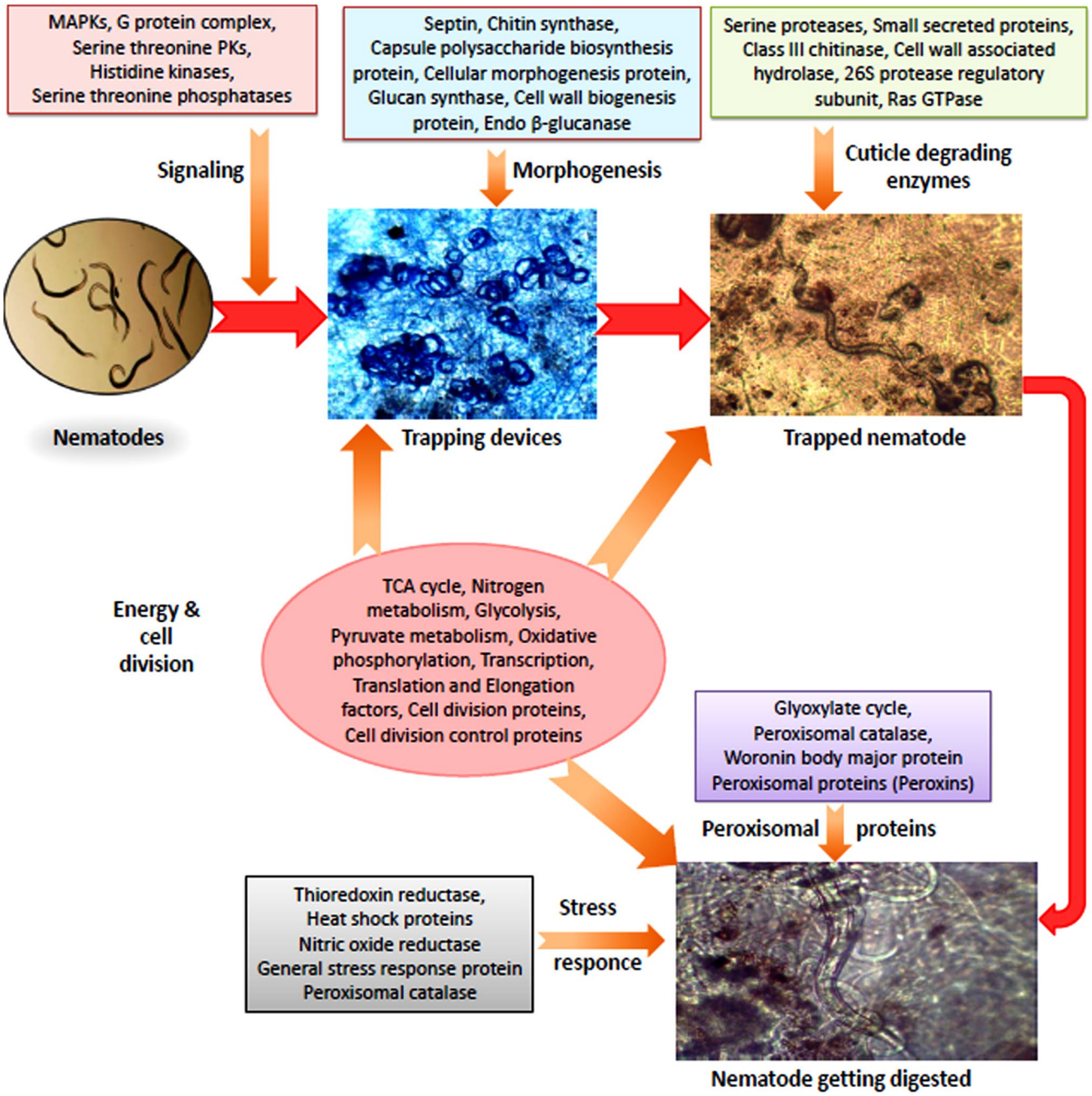


Fig. 9 A proposed model of catching and killing of *Meloidogyne* sp. of root-knot nematodes by *A. conoides*

involved in cell wall synthesis v*iz*., chitin syntheses, chitin synthase activator and glucan synthase were up regulated in induced fungus. In addition, cell wall modifying enzymes, endo-β-glucanase, GPI enchored endo- β-glucanase and endoglucanse-II, which might have role in morphogenesis of trapping devices, were also found up regulated in the induced fungus. Further, a GO:0070879 which is involved in fungal-type cell wall beta-glucan metabolic process was unique to *A. conoides* and its homologs were absent in other nematode-trapping fungi. Astonishingly, a GPI-anchored cell wall β-endo-glucanase which is also a crucial enzyme for cell wall synthesis was down regulated in the induced fungus.

An autophagy related proteins and autophagy regulatory proteins were found up regulated in the presence of nematode extract in *A. conoides*. Autophagy is required for trap formation in *A. oligospora* (Chen et al. 2013). It has been widely accepted that nutrients deprived conditions and presence of nematodes triggers nematophagous fungi for predaceous life, this could be the reason for up regulation

of autophagy proteins in *A. conoides*, as we have used half strength medium during the experiments. This was also supported by enrichment of amino acid transports in *A. conoides* as generally under nutritional deprived conditions nematophagous fungi use nematode as source of nitrogen if they are present in the vicinity. Besides these genes, cofilin cof1, Ras and Rac were also differentially expressed in *A. conoides* when baited with nematode extract. All these proteins have proven role in the morphogenesis, especially in dimorphic fungi (Ballou et al. 2013; Banuett et al. 2008; Berepiki et al. 2011; Fortwendel 2015), suggest their role in morphogenesis and trap formation in *A. conoides* as well. Interestingly, a Myb family of conidiophores development protein was also expressed in *A. conoides* in the presence of nematode extract. Though the data are not presented here, *A. conoides* produced large number of conidiospores when incubated with live nematodes for 24 h. Thus, up regulation of this gene in *A. conoides* explains the production of bulky conidiospores. In addition, capsule polysaccharide biosynthesis protein and capsular associated proteins were differentially expressed in the induced fungus. These proteins may have role in formation of outer adhesive layers on the network of trapping device which aid in trapping the nematodes. A capsular associated protein might have similar function that of AOL protein of *A. oligospora* (Rosen et al. 1992). Septin, a very essential protein associated with cytokinasis, cell polarity, morphogenesis (Alvarez-Tabares and Perez-Martin 2010) was also found up regulated in *A. conoides* in the presence of nematode extract. In addition, several transcription, translation and cell division control proteins were also unregulated in induced fungus. These genes contribute in fungus growth, development and also in nutrient acquisition. All the differentially expressed genes altogether are required for development of trapping devices.

Peroxisomal proteins which are found in dense bodies of trapping structure

Trap cells of nematode-trapping fungi possess electron dense bodies which are common in functions like peroxisome (Dowsett et al. 1977; Heintz and Pramer 1972; Veenhuis et al. 1989). In *A. conoides*, several peroxisome genes, peroxisome biosynthesis protein (pas1), peroxisomal dehydratase, peroxisomal catalase, peroxisomal matrix protein, peroxisomal hydratase-dehydrogenase-epimerase and peroxisomal targeting signal-1 receptor were up regulated in the presence of nematode extract. In addition, a woronin body major protein which are involved in formation of woronin bodies and are fungal peroxisome was also found up regulated in *A. conoides*. Woronin bodies are involved in healing of mycelia and pathogenesis in various plant pathogenic fungi (Beck and Ebel 2013; Soundararajan et al. 2004), as well as also reported for pathogenicity in *A. oligospora* (Yang et al. 2011a). Thus these genes may have similar role in *A. conoides*. Peroxisomal catalase may be in involved in oxidative stress response, pathogenicity and other functions as reported in plant and entomopathogenic fungi (Chantasingh et al. 2013; Paris et al. 2003). Similarly, the malate dehydrogenase, isocitrate lyase and malate synthase, enzymes of the glyoxylate cycle were also found up regulated in induced fungus and glyoxylate cycle has been reported for the fungal virulence (Lorenz and Fink 2001) and also for pathogenesis in *A. oligospora* (Zhao et al. 2014).

Hydrolytic enzymes vital to degrade the host cuticle and other polysaccharides

Once the target nematodes are trapped into the trapping devices, next course of action is to penetrate host cuticle, where cuticle is degraded to acquire the nutrients. Extracellular hydrolytic enzyme plays a decisive role in degradation of exterior cuticle of host, as it is made up of chitin and several proteinous materials (Yang et al. 2007c). Till date, several pathogenesis associated proteases (Braga et al. 2012; Cruz et al. 2015; Nagee et al. 2008; Wang et al. 2009; Yang et al. 2005, 2007a, b, 2011c) and chitinases (Gan et al. 2007; Nguyen et al. 2008; Tikhonov et al. 2002; Yang et al. 2010) have been reported in numerous nematophagous fungi. In *A. conoides*, subtilicin like serine protease, alkaline serine protease, ATP dependent protease la, ADAM-B protease, small secreted protein (SSPs) and cell wall associated hydrolase were up regulated in the presence of nematode extract. We have already confirmed the induction of serine protease gene in our fungus using real time PCR earlier (Pandit et al. 2014a) and in this study too. Small secreted proteins play an imperative role in virulence of plant pathogenic fungi, similar may be its function in *A. conoides*. In addition, a class iii chitinase was up regulated in induced *A. conoides*. Chitinases are involved in the degradation of the egg shell in egg parasitic fungi (Khan et al. 2004; Tikhonov et al. 2002; Van Nguyen et al. 2007). Thus, proteases and chitinases together play a very key role in nematode-trapping fungi, during the process of infection and simultaneous degradation of the nematode cuticle.

Stress responsive and other genes

Owing to their sessile life, fungi have developed sophisticated responses to the diverse environmental stresses (Kroll et al. 2013). As *A. conoides* invade host, it gets exposed to innumerable host metabolites and other bio-molecules which might function as armoury for the defence of host. Stress responsive genes, thioredoxin reductase, hsp70 chaperone, nitric oxide reductase, general stress response protein, stress response rci and multi-sensor hybrid histidine

kinase were found to be up regulated in *A. conoides* in the presence of nematode extract. Other unigenes described earlier are also found to be involved in the stress responses. In a variety of fungi, the adjustment of transcriptome has been reported during invasion into the host (Cairns et al. 2010). The function of oxidative pathways, thioredoxin reductase and nitric oxide reductase to escape oxidative stress is reported in variety of pathogenic fungi (Brown et al. 2009; Missall and Lodge 2005; Thon et al. 2007). So we may conclude that *A. conoides* not only kills and acquires the nutrients from the host but also protects themselves from extraneous environmental stresses. In addition, the differentially expressed hypothetical proteins may have played important roles during the predation, however currently no description is available hence, their functions need to be understood further. Although this study provides new insights and help to understand the fungal parasitism, further studies are required to understand the precise mechanism. Further, the genome information is unavailable for *A. conoides*, the exact gene annotation is bit complex with de novo approach. Hence, in future the genome sequencing and transcriptome analysis at different time interval may help to understand the process in more detail.

Thus, the current data delivers a comprehensive outlook of differentially expressed genes in *A. conoides* both in the presence and absence of the host nematode (*Meloidogyne sp.*) extract. The differentially expressed gene seems to be pathogenicity related genes, often found in *A. oligospora* and plant pathogenic fungi. As *A. conoides* is a dimorphic fungus, it shows its predacious personality, this may be a core reason of sharing more genes with pathogenic fungi, however, not harming plants anyways. Genes of signal transduction pathways including G-proteins, MAPKs and histidine kinases may be initial players to recognise the presence of its prey. Distinct classes of chitinases, capsular polysaccharide biosynthesis protein and genes associated with morphogenesis, which are essential for the development of trapping structures and ultimately to invade inside the host were also up regulated. Similarly, peroxisomal biosynthesis proteins, catalase, oxidase and major enzymes of the glyoxylate cycle which are critical to combat oxidative stress and also involved in pathogenicity were found to be up regulated. Hydrolytic enzymes including subtilicin like serine proteases, class iii chitinase, metalloprotease and small secreted proteins may be involved in digesting nematode cuticle to dig up nutrition from the host. As fungi degrade host cuticle, it is subjected to the oxidative and osmotic stress due to host metabolites and defence machinery. Besides all thioredoxin reductase, nitric oxide reductase, heat shock proteins, molecular chaperone and some additional proteins were differentially expressed to evade this stress. One can also make out the functions of differentially expressed hypothetical genes according to (Shen et al. 2015). Thus, our study enhances the knowledge about the fungal parasitism and will further help to understand and improve the nematode-trapping efficiency, leading to development of superior biocontrol agent against the root-knot nematodes. Still the combined approach of using genomics and proteomics could further help to understand the mechanism of *A. conoides* in detail.

Funding This study was funded by Gujarat State Biotechnology Mission (GSBTM), grant ID: GSBTM/MD/PROJECTS/SSA/3434/2012-13, Gandhinagar, Gujarat, India.

Compliance with ethical standards

Conflict of interest All the authors of the manuscript declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Ahren D, Tunlid A (2003) Evolution of parasitism in nematode-trapping fungi. J Nematol 35(2):194–197

Ahren D, Tholander M, Fekete C, Rajashekar B, Friman E, Johansson T, Tunlid A (2005) Comparison of gene expression in trap cells and vegetative hyphae of the nematophagous fungus *Monacrosporium haptotylum*. Microbiology 151(3):789–803. doi:10.1099/mic.0.27485-0

Al-Hazmi AS, Schmitt DP, Sasser JN (1982) The effect of *Arthrobotrys conoides* on *Meloidogyne incognita* population densities in corn as influenced by temperature, fungus inoculum density, and time of fungus introduction in the soil. J Nematol 14(2):168–173

Alvarez-Tabares I, Perez-Martin J (2010) Septins from the phytopathogenic fungus *Ustilago maydis* are required for proper morphogenesis but dispensable for virulence. PloS ONE 5(9):e12933. doi:10.1371/journal.pone.0012933

Andersson KM, Meerupati T, Levander F, Friman E, Ahren D, Tunlid A (2013) Proteome of the nematode-trapping cells of the fungus *Monacrosporium haptotylum*. Appl Environ Microbiol 79(16):4993–5004. doi:10.1128/AEM.01390-13

Andersson KM, Kumar D, Bentzer J, Friman E, Ahren D, Tunlid A (2014) Interspecific and host-related gene expression patterns in nematode-trapping fungi. BMC Genomics 15:968. doi:10.1186/1471-2164-15-968

Araujo (1998) Predacious activity of *Arthrobotrys* spp isolates on infective *Cooperia punctata* larvae. Braz J Vet Res Anim Sci 35(1):9–11

Ballou ER, Kozubowski L, Nichols CB, Alspaugh JA (2013) Ras1 acts through duplicated Cdc42 and Rac proteins to regulate morphogenesis and pathogenesis in the human fungal pathogen *Cryptococcus neoformans*. PLoS Genet 9(8):e1003687. doi:10.1371/journal.pgen.1003687

Banuett F, Quintanilla RH Jr, Reynaga-Pena CG (2008) The machinery for cell polarity, cell morphogenesis, and the cytoskeleton in the Basidiomycete fungus *Ustilago maydis*-a survey of the genome sequence. Fungal Genet Biol 45(Suppl 1):S3–S14. doi:10.1016/j.fgb.2008.05.012

Beck J, Ebel F (2013) Characterization of the major Woronin body protein HexA of the human pathogenic mold *Aspergillus*

fumigatus. Int J Med Microbiol 303(2):90–97. doi:10.1016/j.ijmm.2012.11.005

Berepiki A, Lichius A, Read ND (2011) Actin organization and dynamics in filamentous fungi. Nat Rev Microbiol 9(12):876–887. doi:10.1038/nrmicro2666

Braga FR, Araújo JV, Soares FE, Geniêr HL, Queiroz JH (2012) An extracellular serine protease of an isolate of *Duddingtonia flagrans* nematophagous fungus. Biocontrol Sci Technol 22(10):1131–1142

Braga FR, Carvalho RO, Silva AR, Araujo JV, Frassy LN, Lafisca A, Soares FE (2014) Predatory capability of the nematophagous fungus *Arthrobotrys robusta* preserved in silica gel on infecting larvae of *Haemonchus contortus*. Trop Anim Health Prod 46(3):571–574. doi:10.1007/s11250-014-0544-2

Brown AJ, Haynes K, Quinn J (2009) Nitrosative and oxidative stress responses in fungal pathogenicity. Curr Opin Microbiol 12(4):384–391. doi:10.1016/j.mib.2009.06.007

Cairns T, Minuzzi F, Bignell E (2010) The host-infecting fungal transcriptome. FEMS Microbiol Lett 307(1):1–11. doi:10.1111/j.1574-6968.2010.01961.x

Catlett NL, Yoder OC, Turgeon BG (2003) Whole-genome analysis of two-component signal transduction genes in fungal pathogens. Eukaryot Cell 2(6):1151–1161

Chantasingh D, Kitikhun S, Keyhani NO, Boonyapakron K, Thoetkiattikul H, Pootanakit K, Eurwilaichitr L (2013) Identification of catalase as an early up-regulated gene in *Beauveria bassiana* and its role in entomopathogenic fungal virulence. Biol Control 67(2):85–93

Chen YL, Gao Y, Zhang KQ, Zou CG (2013) Autophagy is required for trap formation in the nematodet-rapping fungus *Arthrobotrys oligospora*. Environ Microbiol Rep 5(4):511–517. doi:10.1111/1758-2229.12054

Coscarelli W, Pramer D (1962) Nutrition and growth of *Arthrobotrys conoides*. J Bacteriol 84:60–64

Cruz DG, Costa LM, Rocha LO, Retamal CA, Vieira RA, Seabra SH, Silva CP, DaMatta RA, Santos CP (2015) Serine proteases activity is important for the interaction of nematophagous fungus *Duddingtonia flagrans* with infective larvae of trichostrongylides and free-living nematodes *Panagrellus* spp. Fungal Biol 119(8):672–678.

Davies (2005) Interactions between nematodes and microorganisms: bridging ecological and molecular approaches. Adv Appl Microbiol 57:53–78

Degenkolb T, Vilcinskas A (2016a) Metabolites from nematophagous fungi and nematicidal natural products from fungi as alternatives for biological control. Part II: metabolites from nematophagous basidiomycetes and non-nematophagous fungi. Appl Microbiol Biotechnol 100(9):3813–3824. doi:10.1007/s00253-015-7234-5

Degenkolb T, Vilcinskas A (2016b) Metabolites from nematophagous fungi and nematicidal natural products from fungi as an alternative for biological control. Part I: metabolites from nematophagous ascomycetes. Appl Microbiol Biotechnol 100(9):3799–3812. doi:10.1007/s00253-015-7233-6

Dowsett JA, Reid L, Caeseele V (1977) Transmission and scanning electron microscope observations on the trapping of nematodes by *Dactylaria brochopaga*. Can J Bot 55(23):2945–2955

Drechsler (1937) Some hyphomycetes that prey on free-living terricolous nematodes. Mycologia 29(4):447–552

Drechsler (1941) Some hyphomycetes parasitic on free-living terricolous nematodes. Phytopathology 31(9):773–802.

Duddington C (1949) A new predacious species of Trichothecium. Trans Br Mycol Soc 32(3):284–287

Falbo MK, Soccol VT, Sandini IE, Vicente VA, Robl D, Soccol CR (2013) Isolation and characterization of the nematophagous fungus *Arthrobotrys conoides*. Parasitol Res 112(1):177–185. doi:10.1007/s00436-012-3123-3

Fortwendel JR (2015) Orchestration of morphogenesis in filamentous fungi: conserved roles for Ras signaling networks. Fungal Biol Rev 29(2):54–62. doi:10.1016/j.fbr.2015.04.003

Gan Z, Yang J, Tao N, Liang L, Mi Q, Li J, Zhang KQ (2007) Cloning of the gene Lecanicillium psalliotae chitinase Lpchi1 and identification of its potential role in the biocontrol of root-knot nematode Meloidogyne incognita. Appl Microbiol Biotechnol 76(6):1309–1317. doi:10.1007/s00253-007-1111-9

Gotz S, Garcia-Gomez JM, Terol J, Williams TD, Nagaraj SH, Nueda MJ, Robles M, Talon M, Dopazo J, Conesa A (2008) High-throughput functional annotation and data mining with the Blast2GO suite. Nucleic Acids Res 36(10):3420–3435. doi:10.1093/nar/gkn176

Grant CL, Coscarelli W, Pramer D (1962) Statistical measurement of biotin, thiamine, and zinc concentrations required for maximal growth of *Arthrobotrys conoides*. Appl Microbiol 10:413–417

Gronvold J, Wolstrup J, Nansen P, Henriksen SA, Larsen M, Bresciani J (1993) Biological control of nematode parasites in cattle with nematode-trapping fungi: a survey of Danish studies. Vet Parasitol 48(1–4):311–325

Gustin MC, Albertyn J, Alexander M, Davenport K (1998) MAP kinase pathways in the yeast *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev 62(4):1264–1300

Heintz CE, Pramer D (1972) Ultrastructure of nematode-trapping fungi. J Bacteriol 110(3):1163–1170

Hertzberg H, Larsen M, Maurer V (2001) Biological control of helminths in grazing animals using nematophagous fungi. Berl Munch Tierarztl Wochenschr 115(7–8):278–285

Hsueh YP, Mahanti P, Schroeder FC, Sternberg PW (2013) Nematode-trapping fungi eavesdrop on nematode pheromones. Curr Biol 23(1):83–86. doi:10.1016/j.cub.2012.11.035

Kalele DN, Affokpon A, Coosemans J, Kimenju JW (2010) Suppression of root-knot nematodes in tomato and cucumber using biological control agents. Afr J Hortic Sci 3:72–80

Khan A, Williams KL, Nevalainen HK (2004) Effects of *Paecilomyces lilacinus* protease and chitinase on the eggshell structures and hatching of *Meloidogyne javanica* juveniles. Biol Control 31(3):346–352

Khan A, Williams KL, Soon J, Nevalainen HK (2008) Proteomic analysis of the knob-producing nematode-trapping fungus *Monacrosporium lysipagum*. Mycol Res 112(12):1447–1452. doi:10.1016/j.mycres.2008.06.003

Kroll K, Pahtz V, Kniemeyer O (2013) Elucidating the fungal stress response by proteomics. J Proteom 97(31):151–163. doi:10.1016/j.jprot.2013.06.001

Larriba E, Jaime MD, Carbonell-Caballero J, Conesa A, Dopazo J, Nislow C, Martin-Nieto J, Lopez-Llorca LV (2014) Sequencing and functional analysis of the genome of a nematode egg-parasitic fungus, *Pochonia chlamydosporia*. Fungal Genet Biol 65:69–80. doi:10.1016/j.fgb.2014.02.002

Lengeler KB, Davidson RC, D'Souza C, Harashima T, Shen WC, Wang P, Pan X, Waugh M, Heitman J (2000) Signal transduction cascades regulating fungal development and virulence. Microbiol Mol Biol Rev 64(4):746–785

Li J, Zou C, Xu J, Ji X, Niu X, Yang J, Huang X, Zhang KQ (2015) Molecular mechanisms of nematode-nematophagous microbe interactions: basis for biological control of plant-parasitic nematodes. Ann Rev Phytopathol 53:67–95. doi:10.1146/annurev-phyto-080614-120336

Liang L, Wu H, Liu Z, Shen R, Gao H, Yang J, Zhang K (2013) Proteomic and transcriptional analyses of *Arthrobotrys oligospora* cell wall related proteins reveal complexity of fungal virulence against nematodes. Appl Microbiol Biotechnol 97(19):8683–8692. doi:10.1007/s00253-013-5178-1

Liu K, Zhang W, Lai Y, Xiang M, Wang X, Zhang X, Liu X (2014) *Drechslerella stenobrocha* genome illustrates the mechanism of

constricting rings and the origin of nematode predation in fungi. BMC Genomics 15:114. doi:10.1186/1471-2164-15-114
Lorenz MC, Fink GR (2001) The glyoxylate cycle is required for fungal virulence. Nature 412(6842):83–86
Meerupati T, Andersson KM, Friman E, Kumar D, Tunlid A, Ahren D (2013) Genomic mechanisms accounting for the adaptation to parasitism in nematode-trapping fungi. PLoS Genet 9(11):e1003909. doi:10.1371/journal.pgen.1003909
Missall TA, Lodge JK (2005) Thioredoxin reductase is essential for viability in the fungal pathogen *Cryptococcus neoformans*. Eukaryot Cell 4(2):487–489. doi:10.1128/EC.4.2.487-489.2005
Morton OC, Hirsch PR, Kerry BR (2004) Infection of plant-parasitic nematodes by nematophagous fungi – a review of the application of molecular biology to understand infection processes and to improve biological control. Nematology 6(2):161–170
Nagee A, Acharya A, Shete A, Mukhopadhyaya PN, Aich BA (2008) Molecular characterization of an expressed sequence tag representing the cuticle-degrading serine protease gene (PII) from the nematophagous fungus *Arthrobotrys oviformis* by differential display technology. Genet Mol Res 7(4):1200–1208
Nguyen NV, Kim YJ, Oh KT, Jung WJ, Park RD (2008) Antifungal activity of chitinases from *Trichoderma aureoviride* DY-59 and Rhizopus microsporus VS-9. Curr Microbiol 56(1):28–32. doi:10.1007/s00284-007-9033-4
Niu XM, Zhang KQ (2011) *Arthrobotrys oligospora*: a model organism for understanding the interaction between fungi and nematodes. Mycology 2(2):59–78. doi:10.1080/21501203.2011.562559
Oka Y, Koltai H, Bar-Eyal M, Mor M, Sharon E, Chet I, Spiegel Y (2000) New strategies for the control of plant-parasitic nematodes. Pest Manag Sci 56:983–988
Pandit RJ, Bhatt VD, Mukhopadhyaya PN, Joshi CG, P. KA (2014a) Biochemical and molecular characterization of protease from *Arthrobotrys conoides* and *Duddingtonia flagrans*. Int J Adv Biotec Res 5(3):552–561
Pandit RJ, Kunjadia PD, Mukhopadhyaya PN, Joshi CG, H. NA (2014b) Isolation, molecular characterization and predatory activity of two Indian isolates of nematode-trapping fungi. Appl Biol Res 16(1):1–11. doi:10.5958/0974-4517.2014.00042.1
Paris S, Wysong D, Debeaupuis JP, Shibuya K, Philippe B, Diamond RD, Latge JP (2003) Catalases of *Aspergillus fumigatus*. Infect Immun 71(6):3551–3562
Pramer D, Stoll NR (1959) Nemin: a morphogenic substance causing trap formation by predaceous fungi. Science 129(3354):966–967
Robinson MD, Oshlack A (2010) A scaling normalization method for differential expression analysis of RNA-seq data. Genome Biol 11(3):R25
Rosen S, Ek B, Rask L, Tunlid A (1992) Purification and characterization of a surface lectin from the nematode-trapping fungus *Arthrobotrys oligospora*. J Gen Microbiol 138(12):2663–2672
Saxena G et al (1987) Interaction of nematodes with nematophagus fungi: induction of trap formation, attraction and detection of attractants. FEMS Microbiol Lett 45(6):319–327
Schmidt AR, Dorfelt H, Perrichot V (2007) Carnivorous fungi from *Cretaceous amber*. Science 318(5857):1743. doi:10.1126/science.1149947
Schmieder R, Edwards R (2011) Quality control and preprocessing of metagenomic datasets. Bioinformatics 27(6):863–864. doi:10.1093/bioinformatics/btr026
Shen B, Xiao J, Dai L, Huang Y, Mao Z, Lin R, Yao Y, Xie B (2015) Development of a high-efficiency gene knockout system for *Pochonia chlamydosporia*. Microbiol Res 170:18–26. doi:10.1016/j.micres.2014.10.001
Soundararajan S, Jedd G, Li X, Ramos-Pamplona M, Chua NH, Naqvi NI (2004) Woronin body function in *Magnaporthe grisea* is essential for efficient pathogenesis and for survival during nitrogen starvation stress. Plant Cell 16(6):1564–1574. doi:10.1105/tpc.020677
Thon M, Al-Abdallah Q, Hortschansky P, Brakhage AA (2007) The thioredoxin system of the filamentous fungus *Aspergillus nidulans*: impact on development and oxidative stress response. J Biol Chem 282(37):27259–27269. doi:10.1074/jbc.M704298200
Tikhonov VE, Lopez-Llorca LV, Salinas J, Jansson HB (2002) Purification and characterization of chitinases from the nematophagous fungi *Verticillium chlamydosporium* and *V. suchlasporium*. Fungal Genet Biol 35(1):67–78. doi:10.1006/fgbi.2001.1312
Tunlid A, Ahren D (2011) Molecular mechanisms of the interaction between nematode-trapping fungi and nematodes: lessons from genomics. Prog Biol Control 11:145–169. doi:10.1007/978-1-4020-9648-8_6
Van Nguyen N, Kim Y-J, Oh K-T, Jung W-J, Park R-D (2007) The role of chitinase from *Lecanicillium antillanum* B-3 in parasitism to root-knot nematode *Meloidogyne incognita* eggs. Biocontrol Sci Technol 17(10):1047–1058
Veenhuis M, Van Wijk C, Wyss U, Nordbring-Hertz B, Harder W (1989) Significance of electron dense microbodies in trap cells of the nematophagous fungus *Arthrobotrys oligospora*. Antonie Van Leeuwenhoek 56(3):251–261
Wang B, Liu X, Wu W, Li S (2009) Purification, characterization, and gene cloning of an alkaline serine protease from a highly virulent strain of the nematode-endoparasitic fungus *Hirsutella rhossiliensis*. Microbiol Res 164(6):665–673. doi:10.1016/j.micres.2009.01.003
Wang Y, Coleman-Derr D, Chen G, Gu YQ (2015) OrthoVenn: a web server for genome wide comparison and annotation of orthologous clusters across multiple species. Nucleic Acids Res 43(W1):W78–W84. doi:10.1093/nar/gkv487
Westphal A (2011) Sustainable approaches to the management of plant-parasitic nematodes and disease complexes. J Nematol 43(2):122–125
Yang J, Huang X, Tian B, Wang M, Niu Q, Zhang K (2005) Isolation and characterization of a serine protease from the nematophagous fungus, *Lecanicillium psalliotae*, displaying nematicidal activity. Biotechnol Lett 27(15):1123–1128. doi:10.1007/s10529-005-8461-0
Yang J, Li J, Liang L, Tian B, Zhang Y, Cheng C, Zhang KQ (2007a) Cloning and characterization of an extracellular serine protease from the nematode-trapping fungus *Arthrobotrys conoides*. Arch Microbiol 188(2):167–174. doi:10.1007/s00203-007-0233-x
Yang J, Liang L, Zhang Y, Li J, Zhang L, Ye F, Gan Z, Zhang KQ (2007b) Purification and cloning of a novel serine protease from the nematode-trapping fungus *Dactylellina varietas* and its potential roles in infection against nematodes. Appl Microbiol Biotechnol 75(3):557–565. doi:10.1007/s00253-007-0839-6
Yang J, Tian B, Liang L, Zhang KQ (2007c) Extracellular enzymes and the pathogenesis of nematophagous fungi. Appl Microbiol Biotechnol 75(1):21–31. doi:10.1007/s00253-007-0881-4
Yang Y, Yang E, An Z, Liu X (2007d) Evolution of nematode-trapping cells of predatory fungi of the Orbiliaceae based on evidence from rRNA-encoding DNA and multiprotein sequences. Proc Natl Acad Sci USA 104(20):8379–8384. doi:10.1073/pnas.0702770104
Yang J, Gan Z, Lou Z, Tao N, Mi Q, Liang L, Sun Y, Guo Y, Huang X, Zou C, Rao Z, Meng Z, Zhang KQ (2010) Crystal structure and mutagenesis analysis of chitinase CrChi1 from the nematophagous fungus *Clonostachys rosea* in complex with the inhibitor caffeine. Microbiology 156(12):3566–3574. doi:10.1099/mic.0.043653-0
Yang J et al (2011a) Genomic and proteomic analyses of the fungus *Arthrobotrys oligospora* provide insights into nematode-trap formation. PloS Pathog 7:e1002179. doi:10.1371/journal.ppat.1002179

Yang J, Wang L, Ji X, Feng Y, Li X, Zou C, Xu J, Ren Y, Mi Q, Wu J, Liu S, Liu Y, Huang X, Wang H, Niu X, Li J, Liang L, Luo Y, Ji K, Zhou W, Yu Z, Li G, Li L, Qiao M, Feng L, Zhang KQ (2011b) Genomic and proteomic analyses of the fungus *Arthrobotrys oligospora* provide insights into nematode-trap formation. PLoS Pathog 7(9):e1002179. doi:10.1371/journal.ppat.1002179

Yang J, Zhao X, Liang L, Xia Z, Lei L, Niu X, Zou C, Zhang KQ (2011c) Overexpression of a cuticle-degrading protease Ver112 increases the nematicidal activity of *Paecilomyces lilacinus*. Appl Microbiol Biotechnol 89(6):1895–1903. doi:10.1007/s00253-010-3012-6

You FM, Huo N, Gu YQ, Luo MC, Ma Y, Hane D, Lazo GR, Dvorak J, Anderson OD (2008) BatchPrimer3: a high throughput web application for PCR and sequencing primer design. BMC Bioinformatics 9:253. doi:10.1186/1471-2105-9-253

Zhao X, Wang Y, Zhao Y, Huang Y, Zhang KQ, Yang J (2014) Malate synthase gene AoMls in the nematode-trapping fungus *Arthrobotrys oligospora* contributes to conidiation, trap formation, and pathogenicity. Appl Microbiol Biotechnol 98(6):2555–2563. doi:10.1007/s00253-013-5432-6