Integrated Metabolomics and Morphogenesis Reveals Volatile Signaling of the Nematode-Trapping Fungus

Arthrobotrys oligospora

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Running title: Flexible tactics of A. oligospora to trap nematodes
ABSTRACT

The adjustment of metabolic patterns is fundamental to fungal biology and plays vital roles in adaption to diverse ecological challenges. Nematode trapping fungi can switch lifestyles from saprophytic to pathogenic by developing specific trapping devices induced by nematodes to infect their prey as a response to nutrient depletion in nature. However, the chemical identity of the specific fungal metabolites used during the switch remains poorly understood. We hypothesized that these important signal molecules might be volatile in nature. GC-MS was used to carry out comparative analysis of fungal metabolomics during saprophytic and pathogenic lifestyles of the model species *Arthrobotrys oligospora*. Two media commonly used in research on this species, corn meal agar (CMA) and potato dextrose agar (PDA), were chosen in this study. The fungus produced a small group of volatile furanone and pyrone metabolites that were associated with the switch from saprophytic to pathogenic stages. *A. oligospora* grown on CMA tended to produce more traps and employ attractive furanones to improve utilization of traps, while fungus grown on PDA developed fewer traps and used nematodetoxic furanone metabolites to compensate for insufficient traps. Another volatile pyrone metabolite, maltol, was identified as a morphological regulator for enhancing trap formation. Deletion of gene *AOL_s00079g496* in *A. oligospora* led to increased furanone attractant (2 folds) in mutants and enhanced attractive activity (1.5 fold) of the fungus, while resulted in decreased trap formation. This investigation provides new insights regarding the comprehensive tactics of fungal adaptation to environmental stress, integrating both morphological and metabolomic mechanisms.

KEYWORDS: Nematode-trapping fungi; *Arthrobotrys oligospora*; Metabolic adaptation; Pathogenicity; Volatile Organic Compounds (VOCs)
Importance

Nematode-trapping fungi are a unique group of soil-living fungi that can switch from saprophytic to pathogenic lifestyle once in contact with nematodes as a response to nutrient depletion. In this study, we investigated the metabolic response during the switch and the key types of metabolites involved in the interaction between fungi and nematodes. Our findings indicated that *A. oligospora* develop multiple and flexible metabolic tactics corresponding to different morphological responses to nematodes. *A. oligospora* can use similar volatile furanone and pyrone metabolites with different ecological functions to help capture nematodes in the fungal switch from saprophytic to pathogenic lifestyles. Furthermore, *A. oligospora* mutants with increased furanone and pyrone metabolites confirmed the results. This investigation reveals the importance of volatile signaling in the comprehensive tactics used by nematode trapping fungi, integrating both morphological and metabolomic mechanisms.

**INTRODUCTION**

Nematode-trapping fungi (NTF) can detect the presence of nematodes and develop specialized mycelial trap devices to infect and consume prey as a response to nutrient depletion (1-4). These fungi are broadly distributed in terrestrial and aquatic ecosystems, and more than 200 species from the phyla Ascomycota, Basidiomycota, and Zygomycota have been described. Their role as natural enemies of parasitic nematodes makes them attractive as biocontrol agents; moreover their unique ability to switch between saprophytic and parasitic lifestyles are of great interest in basic ecological research (5, 6).

The direct physical contact with living nematodes has been assumed as the crucial biotic factor necessary to induce the trap formation of NTF (7, 8). Traps are regarded as the key morphological indication of the switch from the saprophytic to the pathogenic lifestyle for NTF (9-15). The nematodes not only induce the formation of fungal traps, but once trapped, they also serve as a food source (3). Considerable progress has been made in our understanding of the evolution and molecular mechanisms of fungal trap formation at genomic, proteomic and transcriptomic levels (14, 16). When nematodes
induce the formation of trapping devices, multiple fungal signal transduction pathways are activated and the downstream genes associated with energy metabolism, biosynthesis of the cell wall and adhesive proteins involved in trap formation are regulated (7, 17).

Interestingly, NTF need an organic energy source other than nematodes in order to remain in an active nematophagous state (18, 19). Previous studies have found that corn meal agar (CMA) and potato dextrose agar (PDA) are among the best media for keeping the nematophagous activities of NTF and the most extensively used by experimentalists to observe trap formation induced by nematodes (8, 12, 14, 16, 17, 18). The composition of the growth medium is important because fungi produce different numbers of traps and have different nematocidal activities when grown on CMA or PDA. However, the chemical identity of the signaling molecules responsible for these differential responses has remained unclear.

It has long been assumed that traps are not the only weapons that NTF use to infect nematodes. In 1955, Duddington and Shepherd suggested that NTF could yield an unknown metabolite, “nematotoxin”, to paralyze or kill nematodes because they found that the infected nematodes became inactive before the infection bulb had completely developed (19, 20). Later in 1963, Olthof reported that the filtrates from NTF parasitized nematodes contained the unstable nematode-inactivating substance (21). In 1994, linoleic acid was reported as a putative nematicidal compound from several nematophagous fungi (22). At the same time, a unique class of hybrid oligosporon metabolites found as chemotaxonomic markers were reported from different strains of a model species Arthrobotrys oligospora isolated from The Netherlands (23), Australia (24) and later from China (25, 26). These known metabolites from A. oligospora are non-volatile compounds and exhibit several biological activities, including moderate antibacterial properties, significant autoregulatory effects on the formation of conidiophores and hyphal fusions in A. oligospora (26, 27). However, the chemical identity of the metabolites involved in the lifestyle switch from saprotrophic to pathogenic phases has remained cryptic. We hypothesized that these signals might be volatile in nature and used gas chromatography-mass spectrometry (GC-MS) in our analyses.
A. oligospora is commonly found in soils from diverse ecological habits and has emerged as the model species for nematode-trapping fungi (5, 27). Under limited conditions, A. oligospora can form three dimensional (3D) traps in direct contact with nematodes. In order to obtain information on morphological and metabolic changes in A. oligospora, two common media, CMA and PDA, were used in our analyses to investigate medium-specific metabolic features, as well to illuminate general aspects of A. oligospora metabolism. There has been no previous report about the response of the fungus just before the fungus starts to form predatory traps via the direct physical contact with nematodes, so a non-direct contact bioassay also was performed between the fungus and living/dead nematodes. Dead nematodes were included in the non-direct contact bioassay in order to further evaluate if it could make different responses to the approaching living and dead nematodes. The time-course designs over short-term intervals have provided successive snapshots of the morphological and metabolic status of A. oligospora (model strain 1.1883) in response to a shift from the absence of the nematodes to the presence of nematodes. GC-MS analysis was performed for metabolite profiling to determine similarities and differences in temporal metabolite responses, and we have identified several volatile compounds that exhibit medium-specific responses during the induction of traps in response to the presence of nematodes.

Materials and Methods

Fungal and Nematode Strains, Media, and Treatment Conditions.

A. oligospora model species strain YMF1.01883 (ATCC 24927) was used in the fungus-nematode interaction bioassays and cultured on CMA (corn (Kunming, China) 20 g L\(^{-1}\), agar (Biofroxx, Einhausen, Germany) 15 g L\(^{-1}\)) or PDA (potato (Kunming, China) 200 g L\(^{-1}\), glucose (Solarbio, Beijing, China) 10 g L\(^{-1}\), agar 15 g L\(^{-1}\)). All the bioassays were conducted in 9 cm diameter glass Petri dishes. Inocula of A. oligospora YMF1.01883 were cultured at 28°C on PDA plates for one week. Then one 5 mm diameter disk of the fungus was cut with a sterile cork borer and was inoculated onto either CMA.
or PDA. The cultures were incubated at 28°C until the fungal lawn occupied half of the Petri plate to obtain strong fresh mycelia.

Two treatment bioassays were performed to evaluate the fungal responses during the *A. oligospora*-nematodes interaction: direct physical contact and non-direct contact. *Caenorhabditis elegans* (strain N2) was cultured in oatmeal medium at 22°C for 6-7 days. The fungal strains treated without nematodes on PDA or CMA, were used as controls for both treatment bioassays.

For the non-direct contact bioassay, the bottom of the Petri plate containing the fungal lawn was inverted over a second Petri plate bottom of identical size containing 1 mL solution of mixed stage living nematodes or dead nematodes (29). In this treatment, the fungi and the nematodes shared the same atmosphere but had no direct physical contact. The two half Petri plates were sealed together with Parafilm and then incubated in a dark chamber at 28 °C. For the control group, 1 mL sterile H2O was used in place of the nematode suspension. Half of the live nematodes were submerged for 20 min in 45°C water to prepare dead nematodes. In each treatment the fungus and nematodes were harvested separately at a 6 hour interval for the first 48 hours and at a 24 hour interval for the subsequent 4 days, respectively. In total, four biological replicates were performed for metabolomic analysis.

For the direct contact bioassay, 1 mL mixed-stage living nematode solution (about 3000 nematodes) was directly added in the center of the fungal lawn; 1 mL sterile H2O was used as control. The Petri dishes were sealed with Parafilm and incubated in a dark chamber at 28°C. The fungal lawn was observed and harvested at a 6 hour interval for the first 48 hours and at a 24 hour interval for the subsequent 4 days, respectively. The fungal lawns with nematodes from each time point were extracted with methanol for metabolomic analysis.

**Morphological Analysis.**

For characterization of fungal growth, development and morphological transitions, microscopy was performed according to the protocols outlined previously (16). Observations of the morphological transitions of hyphal fusion to two-dimensional (2D) nets and morphological transitions to three-
dimensional (3D) traps were assessed at a 6 hour interval for first 48 hours and at a 24 hour interval for subsequent 4 days, respectively. The hyphal fusions and 3D traps were evaluated with a binocular microscope (10 x magnification, Olympus, Japan). Seven fields in each fungal culture were picked at random for observation; microscopic counting was repeated three times; and the data obtained were analyzed statistically. Image stacks were processed using Imaris 6.3.1 (Bitplane) to generate images for publication. The mean corrected data for the fungal strains treated with nematodes were obtained from the outcome of the data in the test minus the data in the control group.

**Metabolomic Profiling.**

The metabolomic profiling analysis involved sample extraction, metabolite detection, metabolomic data preprocessing (e.g., metabolite feature extraction, chromatographic peak alignment, data reduction), and statistical analysis. The metabolic profiles were obtained from direct contact and non-direct contact bioassays conducted on two media. Each treatment group consisted of 4 replicates and a corresponding control group with same number replicates. The fungal mycelial lawn were harvested and extracted twice with 30 mL methanol under ultrasonic conditions for 30 min in an ice-cooled bath-type sonicator. Each methanol-soluble extract was centrifuged for 3 min at 10 000 x g and 4°C and the supernatant was concentrated to dryness under vacuum. Each dried extract was resuspended in 1 mL methanol under ultrasonic conditions for 20 min in an ice-cooled bath-type sonicator, and then filtered through 0.22 μm membranes. The filtrates were stored at -80°C prior to GC-MS analyses.

GC-EI-MS analyses were performed as described (30) using a Hewlett-Packard gas chromatograph 5890 series II Plus linked to a Hewlett-Packard 5972 mass spectrometer system (Hewlett-Packard, San Diego, CA, USA) equipped with a 30 m long, 0.25 mm i.d., and 0.5 μm film thickness HP5-MS capillary column. The temperatures were programmed from 100 to 300 °C at a rate of 5°C/min. Helium was used as a carrier gas at a flow rate of 0.7 mL/min. The split ratio was 1:20, the injector temperature 280 °C, the interface temperature 300 °C, and the ionization voltage 70 eV.
Identification of peaks was performed through retention time index and mass spectrum. Compounds from the strains were designated as metabolites if they were identified with a match 900 on a scale of 0 to 1,000 and retention index (RI) deviation of 3.0 (31, 32). The semiquantitative analysis of the main compounds was performed through internal normalization with the area of each compound. The addition of each area of the compounds corresponds to 100% area (33).

Data Analysis.

The data matrix was analyzed by Principal Component Analysis (PCA (34). The principle component calculations were performed using TIGR MultiExperiment Viewer (MeV) software with a Centering Mode, based on means, and visualized by using the Eigenvalues of the first principal component (x-axis) and the second principal component (y-axis) or second principal component (x-axis) and the third principal component (y-axis) (34). Each point on the plot represents an individual sample, and each point on the loading plot represents a contribution of an individual metabolite to the score plot. Accordingly, chemical components responsible for the differences between samples detected in the scores plot can be extracted from the corresponding loadings.

Samples were clustered using unsupervised hierarchical cluster analysis (HCA) that provides organization of primary data sets without predefined classification. Data were visualized by dendrograms. Logarithmized values of metabolite relative concentrations were implemented in TIGR Mev software in an unsupervised hierarchical cluster analysis (HCA) using Pearson correlation (34, 35).

Chemotaxis and Nematodetoxic Assays.

In order to evaluate if these volatile metabolites and mutants have nematode-attracting ability, chemotaxis assays were preformed in 9 cm plates containing assay medium (20% agar, 5 mM potassium phosphate pH 6.0, 1 mM CaCl₂, 1 mM MgSO₄) according to published protocols (36, 37). Two marks, at opposite ends, were made on the back of the Petri plate, about 1 cm from the edge of the plate. Between 100 and 200 washed adult nematodes were placed near the center of a 9 cm assay plate with the putative attractant at one end of the plate and an aliquot of 1µL solvent ethanol was placed over the
other mark as control. An aliquot containing each respective test compound was suspended in 1µL of ethanol and placed on the agar over one mark. Test compounds, including propanoic acid (1), 3-ethoxy-1,2-propanediol (2), 2(5H)-furanone (5), furan-2-ylmethanol (6), furan-2-carbaldehyde (7), 5-methylfuran-2-carbaldehyde (8), and n-Hexadecanoic acid (13), (Z,Z)-9,12-methyl octadecadienoate (14) were obtained from Sigma-Aldrich USA, and D-(+)-Talose (12) was obtained from TCI Tokyo Chemical Industry Co., Ltd. Japan. To evaluate the mutant strains, a 6 mm diameter disk of mycelium grown for 2 days on CMA medium was used as test sample. For negative controls, a 6 mm diameter disk of the wildtype strain on CMA medium was used. About 100 washed *C. elegans* adult nematodes in M9 buffer were placed near the center of the plate, equidistant from the two marks. After 1hr, the number of *C. elegans* at the putative attractant area and at the control area was counted. A chemotaxis index was calculated based on the enrichment of animals at the attractant as following formula:

\[
\text{chemotaxis index} = \frac{\text{the number of nematodes at the attractant area} - \text{the number of nematodes at the control}}{\text{the total number of the Nematodes}}.
\]

The chemotaxis index varied from +1.0 to -1.0. In this assay, a chemotaxis index of 1.0 represents complete preference for the test sample, and an index of 0 represents an equal distribution.

The nematode toxicity test was performed according to a previously published protocol (38). About 300 *C. elegans* were dispensed into 3.5 cm plates containing 1 mL of M9 buffer with variable amounts of pure metabolites (dissolved in DMSO) per plate. The same volume solvent of DMSO (0.5% DMSO, v/v) was used as a negative control group and 1 µg/mL ivermectin (Sigma-Aldrich USA) was used as a positive control. Worms were exposed for 24 h at 20 °C, and the number of dead or living worms was determined by the absence/presence of touch-provoked movement when probed with a platinum wire. The median lethal concentration (LC50) value was calculated using the probit method (38). All treatments were conducted in triplicate.

**Mutant Construction.**

The annotation of the genome of *A. oligospora* revealed five putative PKS genes including...
The gene \textit{AOL_s00043g287} encodes a type III PKS and is designated \textit{PKS III-1}. The genes \textit{AOL_s00043g828}, \textit{AOL_s00079g496}, \textit{AOL_s00215g283}, and \textit{AOL_s00215g926} encode type I PKSs and are designated \textit{PKS I-1}, \textit{PKS I-2}, \textit{PKS I-3}, and \textit{PKS I-4}, respectively. A modified protoplast transformation method (30) for genetic disruption of these PKS genes was applied using double-crossover recombination with the hygromycin-resistance gene (\textit{hyg}) as a selection marker, followed by identification of desired mutants using diagnostic PCR. The two homologous regions were amplified from \textit{A. oligospora} genomic DNA using primers containing overlapping regions with the vector pAg1-H3 and the \textit{hyg}-resistance cassette.

Genomic DNA of \textit{A. oligospora} was extracted as previously described (16). Restriction endonucleases and DNA modifying enzymes were purchased from New England Biolabs (Beverly, MA). In-Fusion\textsuperscript{®} HD Cloning Kits were purchased from Clontech Laboratories (Mountain View, CA).

The left and right DNA fragments flanking the hygromycin resistant gene (\textit{hygR}) in pAg1-H3 vector were amplified from the genomic DNA of \textit{A. oligospora} by PCR (GXL high-fidelity DNA Polymerase TaKaRa Biotechnology Co. Ltd, Dalian, China) using primer sets as following. The disruption vector for PKS III-1 gene \textit{AOL_s00043g287} was constructed with primer sets: 287-5f (TCGAGCTCGGTACCAAGGCCCGGGTAAGACGTTAGGGGCTGC), 287-5r (GAGGCCTGATCATGGGCCCGGACTTACGACTGGGACT), 287-3f (GCGATCCGCCGCGCCGCCGGCCGGCCGAGGCTTCTTCTGGAAA) and 287-3r (GAGTCACGAAGCTTGCATGCTGAGGTGTGCGGCTTGCTTGTA).

The disruption vector for PKS I-1 gene \textit{AOL_s00043g828} was constructed with primer sets: 828-5f (GAGCTCGGTACCAAGGCCCGGGTGCGTCACTTTGTTCATC), 828-5r (CGAGGCCTGATCATGGGCCCGGACTTACGACTGGGACT), 828-3f (GCGATCCGCCGCGCCGCCGGCCGGCCGAGGCTTCTTCTGGAAA) and 828-3r (GAGTCACGAAGCTTGCATGCTGAGGTGTGCGGCTTGCTTGTA).

The disruption vector for PKS I-2 gene \textit{AOL_s00079g496} was constructed with primer sets: 496-5f (TCACGAAGCTTGCATGCTGAGGTGTGCGGCTTGCTTGTA). The disruption vector for PKS I-3 gene \textit{AOL_s00215g283} was constructed with primer sets: 283-5f (TCGAGCTCGGTACCAAGGCCCGGGTAAGACGTTAGGGGCTGC), 283-5r (GAGGCCTGATCATGGGCCCGGACTTACGACTGGGACT), 283-3f (GCGATCCGCCGCGCCGCCGGCCGGCCGAGGCTTCTTCTGGAAA) and 283-3r (GAGTCACGAAGCTTGCATGCTGAGGTGTGCGGCTTGCTTGTA).

The disruption vector for PKS I-4 gene \textit{AOL_s00215g926} was constructed with primer sets: 926-5f (TCGAGCTCGGTACCAAGGCCCGGGTAAGACGTTAGGGGCTGC), 926-5r (GAGGCCTGATCATGGGCCCGGACTTACGACTGGGACT), 926-3f (GCGATCCGCCGCGCCGCCGGCCGGCCGAGGCTTCTTCTGGAAA) and 926-3r (GAGTCACGAAGCTTGCATGCTGAGGTGTGCGGCTTGCTTGTA).
AGCTCGGTACCAAGGCCCGGGTTTTATAGAAATGCCCTCC and 496-5r: 248
GAGGCCTGATCATCGATGGGCCCGTCTTACCCAACTTAGCG, and 496-3f:
GCGATCGCCGCGCCGCGCCGCGCCAGATAGTAAGGATGGGCAG and 496-3r:
TCACGAAGCTTTGCTCAGGTAAACGCACAGGTTA. The disruption vector for PKS I-3 gene AOL_s00215g283 was constructed with primer sets: primer sets 283-5f, 283-5r, 283-3f and 283-3r (30). The disruption vector for PKS I-4 gene AOL_s00215g926 was constructed with primer sets: 926-5f (GAGCTCGGTACCAAGGCCCGGGCTTACCCAACTTAGCG), 926-5r (AGGCCTGATCATCGATGGGCCCGTCTTACCCAACTTAGCG), 926-3f (TCTAGAGGTCCACTGCTGGCGTTCGTAGTGATG) and 926-3r (CACGAAGCTTGCTACCCACTTTCCAGGTAGGACCGTGA).

The DNA fragments (5' flanks and 3' flanks) were purified using PCR Clean-up Kit (Macherey-Nagel Inc, Düren, Germany) and NucleoSpin Gel, and were inserted into the specific sites of pAg1-H3 vector, respectively, by In-Fusion method to generate the completed disruption pAg1-H3-5'-3' vector. The homologous fragment amplifications were carried out as follows. Twenty-five μL PCR amplification system, using GXL high-fidelity DNA polymerase following the manufacturer’s instructions (Takara) was applied. Half of one microliter of the prepared genomic DNA from A. oligospora was added as template. All PCRs were performed in a Veriti 96-well thermal cycler (Applied Biosystems, Foster city, CA). The amplification program contained pre-denaturation at 98°C for 4 min followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 57°C for 15 s, and elongation at 68°C for 2 min, with a final extension step at 68°C for 10 min.

Medium PDASS (PDA supplemented with 0.6 M sucrose, 0.3 g/L yeast extract, 0.3 g/L tryptone, 0.3 g/L peptone, and 200 μg/mL hygromycin B (Roche Applied Science, Mannheim, Germany) for selecting transformants) was applied to carry out protoplast regeneration. Four 1-1.2 cm diameter mycelia plugs from 7 d fungal strain on YMA medium (2 g/L yeast extract, 10 g/L malt extract, and 18 g/L agar) were inoculated into 100 mL of TG medium [1% tryptone (Oxoid, Basingstoke, U.K.), and 1% glucose] and cultured at 30 °C at 180 rpm for 36 h. The mycelia were harvested and resuspended in
20 mL of a filter-sterilized enzyme solution that contained 120 mg of lysing enzymes (Sigma, St. Louis, MO), 0.4 mL of cellulase (Sigma, St. Louis, MO), and 100 mg of snailase (Solarbio, Beijing, China) in 0.6 M MgSO$_4$ at pH 6.0. The suspension was incubated for 4 h at 28 °C on a rotary shaker at 180 rpm. Protoplasts were collected by filtering through six layers of sterile lens-cleaning tissue and centrifuged at 1000 g. The protoplasts were washed twice with KTC (1.2 M KCl, 10 mM Tris-HCl, 50 mM CaCl$_2$) solution and finally resuspended in the same solution.

The protoplast-based protocol for the disruption of the targeted genes in *A. oligospora* was performed as described previously (16). About 150 μL protoplasts (circa 8.0×10$^7$/mL) were mixed with 10 μg linear DNA in a 1.5 mL centrifuge tube. After 30 min of incubation on ice, 600 μL of PTC (50 mM CaCl$_2$, 20 mM Tris-HCl, 50% polyethylene glycol 6000, pH 7.5) was added into the mixture and mixed gently. After incubation at 28 °C for 1 h, regeneration for 12 h, the putatively transformed protoplasts were plated onto PDAS medium (PDA supplemented with 5 g/L molasses, 0.6 M saccharose, 0.3 g/L yeast extract, 0.3 g/L tryptone, and 0.3 g/L casein peptone) containing 200 μg/mL of hygromycin B. Transformation colonies were selected after incubation at 28 °C for 6-8 d, and every single colony was transferred to a new plate containing TYGA medium (10 g/L tryptone, 10 g/L glucose, 5 g/L yeast extract, 5 g/L molasses, 18 g/L agar) containing 200 μg/mL of hygromycin B. After incubation for 5 d at 28 °C, genomic DNA of putative transformants were extracted and were verified by PCR to check for the integration of genes in the genome. Five mutants deficient in these PKS genes, respectively, were screened out and confirmed by PCR. Knockout of the PKS I-2 gene *AOL_s00079g496* was further confirmed by southern blot analysis. Southern analysis was carried out according to the instructions provided by the Chemiluminescent Nucleic Acid Detection Module (Thermo, Rockford, USA). The primer pair KS-5f (TGTATTCCGTTTCTCCTTGTC) and KS-3r (TTGAACCAACACGATTCGTC) were used as Southern hybridization probes, and restriction enzyme Age I was used to digest the genomic DNA of the wild-type *A. oligospora* and the mutant ∆*AOL_s00079g496* for Southern analysis. All the mutants were maintained and cultured on the same media in the same way as the wildtype strain. The metabolites from cultures of these mutants and the wildtype strain were extracted and analyzed by
HPLC and GC-MS methods. GC-MS analysis was performed as described above in Metabolomic Profiling.

HPLC Analysis.

HPLC analysis was carried out using a HP 1200 unit (Agilent, Waldbronn, Germany), employing the following instrumental conditions: column, CAPCELL PAK C18, 5 μm; 4.6 x250 mm (Shiseido, Tokyo, Japan); mobile phase A, 0.1% formic acid in water; mobile phase B, 0.1% formic acid in acetonitrile. The LC conditions were performed as described previously (30) and were manually optimized on the basis of separation patterns as gradient program of B: 0 min, 10% B; 2 min, 10% B; 10 min, 25% B; 30 min, 35% B; 35 min, 50% B; 45 min, 90% B; 47 min, 10% B; 49 min, 10% B. UV spectra were recorded at 220-400 nm.

RESULTS

Differences in hyphal morphogenesis of A. oligospora on CMA and PDA in response to nematodes

In the non-direct contact bioassay, living and dead nematodes were used to evaluate if the fungus had different morphological and metabolic responses. The morphological responses of A. oligospora grown on CMA and PDA to the presence of nematodes under two modes of contact in 144 h were evaluated and found to be significantly different (Fig. 1). In direct contact with nematodes, the fungal strains grown on both PDA and CMA developed 3D traps. In our study, within 6 h on CMA, the formation of 3D traps was observed, while on PDA the formation of 3D traps was not observed until 12 h. Not only did the fungus on CMA produce traps in a shorter time, after 24 h, fungal strains grown on CMA had more traps than those on PDA. After 30 h, when exposed to nematodes, the fungus cultivated on CMA produced the 3D traps at a level of 100 cm², while the fungal strains grown on PDA formed fewer than half this number of traps. The fungus grown on PDA took 12 hours longer to develop 3D traps at near 80 cm² which is 20% fewer than that of the fungal strains on CMA (Fig. 1).
The non-direct contact bioassay was performed according to published protocols (29). The bottom portion of two glass Petri plates of identical size were used, one containing the fungal culture, the other containing *C. elegans* nematodes. The fungal plate was inverted over the plate containing the nematodes, but there was no direct contact between the worms and the fungal mycelium. Under this condition of non-direct exposure to nematodes, at 24 h, no obvious morphological transition was observed in the fungi cultured on either CMA or PDA (Fig. 1). However, hyphal fusions were observed. A morphological transition of hypha fusions was observed at 30 h for the fungi grown on CMA and at 42 h on PDA. On CMA, the fungal strains developed 30% more hyphal fusions than on PDA (Fig. 1).

Interestingly, the numbers of the hyphal fusions produced by the fungus grown on CMA reached the maximum at 80 cm$^2$ within 96 h, and then quickly decreased to 30 cm$^2$. However, no such change in rate of trap formation was observed for the fungal strains grown on PDA. The numbers of hyphal fusions produced by the strains grown on PDA increased steadily and reached 80 cm$^2$ at the end of the observation period. Exposure to living or dead nematodes made no obvious difference in the formation of hyphal fusions when the fungus was grown on CMA. However, on PDA about 20% more hyphal fusions were observed with exposure to live nematodes than with exposure to dead ones (Fig. 1). The formation of 3D traps was not observed for the fungi grown on CMA until 72 h; 3D traps were observed on PDA after 96 h. Then, while the numbers of 3D traps on both media increased slowly, they remained at a low level (Fig. 1). In summary, the fungi grown on CMA developed more traps, and did so at a faster rate, than those grown on PDA. In the absence of direct contact with nematodes, the fungi grown on either CMA or PDA medium developed more hyphal fusions than 3D traps.

**Metabolites from *A. oligospora* grown on CMA or PDA during the time course between the saprophytic and pathogenic stages**

Time course metabolite profiles of *A. oligospora* YMF1.01883 grown on CMA and PDA treated with nematodes, including direct contact and non-direct contacts with live and dead nematodes, and treated without nematodes were analyzed with GC-MS analysis (Tables S1-S2). In non-direct contact bioassay,
nematodes were also collected at regular intervals for GC/MS analyses in order to remove the effect of the nematodes on *A. oligospora* in direct contact bioassay. Four replicates for each treatment on one medium at one time point led to in total 382 fungal samples and 60 nematode samples for metabolite analysis. At one time point, the metabolite profiles of the fungal strains treated with nematodes were compared with that of the fungal strains treated without nematodes to evaluate the metabolites varying in contents. In order to get more information about the potential metabolites, the peaks were designated as metabolites if they were identified with a match 700 on a scale of 0 to 1,000 to that data in the inborn library. All the metabolites which showed significant changes in concentrations during the time course metabolite profiles were considered (Tables S3-S4).

The metabolite profiles of *A. oligospora* on CMA and PDA, respectively, under four treatments at 24h were analyzed (Fig. S1). It was obvious to note that despite four types of treatments, the fungal strains on the same medium shared quite similar metabolite patterns. It seemed that direct contact between nematodes and fungi did not make an obvious difference in the fungal metabolite profiles. It is also clear that the metabolite profiles of the fungi grown on CMA were different from those grown on PDA. Comparison with the corresponding control groups without nematodes revealed that 34 out of 70 metabolites from the strains on CMA medium and 16 out of 80 metabolites from those on PDA medium were significantly up- or down-regulated during the time course profiles of the fungal contacts with nematodes. Among 34 varying metabolites detected from the fungal strains on CMA, 18 metabolites were found from all the three groups treated with nematodes, 13 metabolites from the group treated with nematodes in direct contact, 2 metabolites from the group treated with live nematodes in non-direct contact, and 1 metabolite from the group treated with dead nematodes in non-direct contact. Among 16 varying metabolites detected from the fungal strains on PDA, 9 metabolites were found from all the three groups treated with nematodes, 5 metabolites from the group treated with nematodes in direct contact, 1 metabolite from the group treated with live nematodes in non-direct contact, and 1 metabolite from the group treated with dead nematodes in non-direct contact. These metabolites included short
Analysis of metabolic patterns of *A. oligospora* grown on CMA and PDA between the saprophytic and the pathogenic stages

The profiled metabolite data were analyzed using principal component analysis (PCA). PCA is a useful clustering method for exploratory data analysis and requires no previous knowledge of data structures. The PCA score trajectories of logarithmically transformed metabolite concentrations from *A. oligospora* during saprophytic and the pathogenic stages, growing on CMA and PDA, are depicted (Fig. 2). These data points are clustered into two distinct groups in the plot maps (Fig. 2A and 2B), indicating clear differences in the fungal extract metabolome between fungi growing on the two different media. The first two principal components account together for 90.2% of the variance. Overall, the first principal component mainly reflected differences in media.

The PCA plots of dendrograms from experiments and metabolite data in CMA and in PDA, respectively, are depicted in Fig. 2C and 2D. The metabolic profiles of the CMA groups displayed more extensive responses to nematodes than the PDA groups, compared with their corresponding time-series controls. A notable transformation of metabolic changes was observed in the CMA groups treated with nematodes under the two different modes of contact. During the time courses of fungal strains cohabitating with nematodes under two different modes of contact, the PCA plot of the metabolites of fungi grown on CMA shows that the experimental groups separate into four main branches: 1) the control branch of *A. oligospora* cohabiting without nematodes (C); 2) the branch of *A. oligospora* cohabiting under direct contact with nematodes (DC); 3) the branch of *A. oligospora* cohabiting under non-direct contact with live nematodes (NDC-L); and 4) the branch of *A. oligospora* cohabiting under non-direct contact with dead nematodes (NDC-D) (Fig. 2C). The fungal strains grown on CMA medium had different metabolic responses not only to the approach and the access of nematodes, but also to the presence of living or dead nematodes. In contrast, on PDA, no obvious distribution of metabolite data in...
the PCA plot was observed with either the modes of contact or the viability status of nematodes (Fig. 2D).

Hierarchical clustering was applied to organize the metabolites based on their relative levels across samples and to discern linkages between these metabolites (Fig. 3). A subset of small molecular metabolite categories, including 11 metabolites in the CMA group and 9 metabolites in the PDA group, were significantly changed while the fungi cohabited with nematodes from 6 h to 96h under both media. Among these metabolites, 6 metabolites in the CMA group consistently changed patterns during the time course. These were propanoic acid (1), 3-ethoxy-1,2-propanediol (2), 6-methoxy-9H-purin-2-amine (3), Hexahydro-2,6-epoxyfuro[3,2]-3-ol (4), 2(5H)-furanone (5), and furan-2-ylmethylanol (6) (Fig. 4). On PDA medium, 7 metabolites showed changing patterns during the time course metabolite profiles. These were furan-2-carbaldehyde (7), 5-methylfuran-2-carbaldehyde (8), 2H-pyran-2,6(3H)-dione (9), 3-hydroxy-2-methyl-4H-pyran-4-one (10), (R)-1-phenyl-1,2-ethanediol (11), D-(+)-Talose (12), n-hexadecanoic acid (13), and (9Z,12 Z)-methyl octadeca-9,12-dienoate (14, methyl ester of linoleic acid) (Fig. 4). These compounds may have potential functional roles in the interaction between the fungal strains and nematodes.

Characterization of the target metabolites during the fungus-nematode interaction.

The roles of 12 of the 14 individual metabolites were evaluated using 12 commercially available compounds, including propanoic acid (1), 3-ethoxy-1,2-propanediol (2), 6-methoxy-9H-purin-2-amine (3), 2(5H)-furanone (5), furan-2-ylmethylanol (6), furan-2-carbaldehyde (7), 5-methylfuran-2-carbaldehyde (8), 3-hydroxy-2-methyl-4H-pyran-4-one (10), (R)-1-phenyl-1,2-ethanediol (11), D-(+)-Talose (12), n-hexadecanoic acid (13), and (9Z,12 Z)-methyl octadeca-9,12-dienoate (14). The use of chemical standards allowed us to test the ability of individual compounds to attract or poison nematodes, as well as to observe their effects on fungal development and morphology.

During the preliminary chemotaxis bioassay, among the metabolites tested at a concentration of 1mg/mL, 0.1 mg/mL, and 0.01mg/mL C. elegans worms were attracted toward only three metabolites:
2(5H)-furanone (5), furan-2-yl methanol (6) and furan-2-carbaldehyde (7). Interestingly, these three metabolites all share a furan ring and have similar molecular weights. Compounds 5 and 6 were characterized from the fungus on CMA, while compound 7 was characterized from the fungus on PDA, based on the time course metabolic profiles of the fungal strains (Fig. 4). To characterize the chemotaxis responses to these three volatile attractants further, worms were tested at concentrations of 1000, 500, 250, 100, 50, 25, 10, 5, and 1 µg/mL, and a chemotaxis index was calculated based on the enrichment of animals at the attractant. The chemotaxis index could vary from 1.0 (perfect attraction) to -1.0 (perfect repulsion). Weakly attractive ethanol was used as the control. The metabolite 2(5H)-furanone (5) functioned as an attractant through a broad range of concentrations, displaying the strongest nematode-attracting ability at a concentration of 250 µg/mL (Fig. 5A). Furan-2-yl methanol (6) showed a more complex response, being attractive when undiluted but somewhat repulsive at low concentrations.

Among the 12 metabolites tested for their toxicity towards *C. elegans*, 5-methylfuran-2-carbaldehyde (8) showed toxic activity against nematodes with a LC₅₀ value of 369 µg/mL in 12 h. The other compounds tested did not display obvious toxic effects at the concentrations tested in these experiments (Fig. 5B).

The same 12 compounds were applied to the fungal cultivation media. In comparison with the solvent control, fungal strains treated with 2.5 µg/mL of 3-hydroxy-2-methyl-4H-pyran-4-one (10), also known as maltol (10), displayed a significant increase in the formation of 3D traps induced by nematodes. Over 12 h, the number of adhesive 3D traps formed by the fungus grown on the media treated with maltol (10) was 189 cm⁻² (Fig. 5C), or 30% more than control untreated media (142 cm⁻²).

Functional validation of the furanone and pyrone metabolites during the fungus-nematode interaction.

Bioinformatics analysis of the *A. oligospora* genome revealed five putative polyketide synthase (PKS) genes including a type III PKS synthase gene *AOL_s00043g287 (PKS III-1)*, and four type I PKS synthase genes *AOL_s00043g828 (PKS I-1), AOL_s00079g496 (PKS I-2), AOL_s00215g283 (PKS I-3),*
and the other \textit{AOL}_s00215g926 (PKS I-4) (30). Disruption of these five PKS genes were performed and five mutants \textit{AOL}_s00043g287 (PKS III-1), \textit{AOL}_s00043g828 (PKS I-1), \textit{AOL}_s00079g496 (PKS I-2), \textit{AOL}_s00215g283 (PKS I-3), \textit{AOL}_s00215g926 (PKS I-4) were screened from 20, 7, 8, 22, and 23 transformants, respectively, by genomic DNA isolation and diagnostic PCR (see Fig. S2 and Fig. S3). The metabolites from cultures of these mutants and the wildtype strain were extracted and analyzed by HPLC and GC-MS methods according to the standard protocols (30). In the HPLC profiles, the mutants \textit{AOL}_s00215g283 (PKS I-3) and \textit{AOL}_s00215g926 (PKS I-4) lacked most of the peaks with retention times ranging between 21 and 40 min (see Table S8). Mutant \textit{AOL}_s00043g287 (PKS III-1) displayed the same HPLC and GC-MS profiles as the wildtype strain. Mutant \textit{AOL}_s00043g828 (PKS I-1) showed three peaks that were not observed in wild type at retention times at 11.68, 17.87 and 18.82 min in the HPLC profile, and then characterized as non-furanone and non-pyrene metabolites by comparison with the standard samples, and further GC-MS analysis. Only the HPLC profile of mutant \textit{AOL}_s00079g496 (PKS I-2) displayed an obvious difference in the peak for the attractant compound 2(5\textit{H})-furanone (5), while most of other peaks were similar to the wild type profile (Fig. 6). It was interesting to note that even grown on PDA, the mutant \textit{AOL}_s00079g496 (PKS I-2) yielded 200\% higher 2(5\textit{H})-furanone (5) than the wildtype strain (Fig. 6). Further chemotaxis bioassays performed on CMA also revealed that 150\% more worms were attracted to \textit{AOL}_s00079g496 (PKS I-2) than to the wildtype strain (Fig. 7), strongly confirming the nematode-attracting function of 2(5\textit{H})-furanone (5).

Mutant \textit{AOL}_s00079g496 (PKS I-2) showed the same growth rates and conditions as the wild-type strain both on CMA and on PDA within 6 days. However, from 9 days on, \textit{AOL}_s00079g496 grown on PDA displayed much fluffier aerial mycelia than the wildtype (Fig. 8). In addition, \textit{AOL}_s00079g496 showed increased spore formations but decreased germination rates than the wild type strain (Fig. 7). When nematodes were added to 4 day PDA cultures, it was surprising to note that \textit{AOL}_s00079g496 formed fewer traps than the wild-type strain (Fig. 7). After 24 h, the number of
adhesive traps formed by ΔAOL_s00079g496 was 69 cm$^{-2}$ with 10% fewer than the number formed by the wildtype strain (77 cm$^{-2}$) (Fig. 7). Accordingly, the number of nematodes infected by the traps produced by the mutant ΔAOL_s00079g496 was 35% fewer than that of the wildtype strain (Fig. 7). Nevertheless, in both the wild type and the mutant strains, all the nematodes were dead within 36 h, despite the fact that ΔAOL_s00079g496 made fewer traps. Further GC-MS analysis revealed that ΔAOL_s00079g496 (PKS I-2) produced more furanone and pyrone metabolites including furan-2-ylmethanol (6), furan-2-carbaldehyde (7), 1-(furan-2-yl)propan-1-one (an ethyl derivative of 7), 5-(hydroxymethyl)furan-2-carbaldehyde (a hydroxy derivative of nematicidal 8), and 3,5-dihydroxy-6-methyl-2H-pyran-4(3H)-one (a hydroxy derivative of 10) than the wild type strain (Fig. 9).

**DISCUSSION**

Nematode trapping fungi have fascinated scientists for decades and many earlier workers have observed the way in which the presence of nematodes alters the morphology and metabolism of trap forming species. Although earlier studies detected attractant and nematocidal metabolites by their activities, the compounds were never chemically identified (21-28). Therefore, we hypothesized that these signaling molecules might be volatile in nature. In our analyses, we used GC-MS and were able to separate and chemically characterize the metabolites, as well as elucidate their biological activities in attracting nematodes, in inducing trap formation, or in killing nematodes.

Under direct physical contact with nematodes, fungi grown on CMA produced more 3D traps than those grown on PDA and did so at a faster rate. Similar results were obtained in the non-direct contact bioassay, however, instead of 3D trap formation, fungal hypha fusions were observed in the non-direct assay. The fungi grown on CMA developed more hyphal fusions and 3D traps than those on PDA. Only at a late stage after 72 h on CMA and 96 h on PDA were a few 3D traps observed. Previous studies suggested that trap formation also requires a hyphal fusion event during initial stages (39), and hyphal fusions were regarded as defensive structures of nematode-trapping fungi (5, 26). This might indicate that in the face of the approaching nematodes, the fungus first moved into a defensive posture through...
hyphal fusion before trap formation induced by direct contact with nematodes. Both bioassays revealed that A. oligospora grown on CMA displayed greater morphological transitions in response to the presence of nematodes than when it was grown on PDA.

The time course of metabolite profiling indicated that the growth medium influenced metabolism more profoundly than the mode of contact with nematodes. When grown on CMA, almost half of (48%) the total detected metabolites changed in response to physical or indirect contact with nematodes; when grown on PDA only 11% of metabolites displayed significant abundance changes in response to direct or indirect contact with nematodes. The fungal strains grown on CMA medium had more extensive metabolic responses to nematodes compared with those grown on PDA medium. The PCA plot of metabolite data from fungal strains on CMA clustered into four main branches, corresponding with the experimental treatments according to the modes of contact (direct or indirect) and the status of nematodes (living or dead). In other words, the fungal strains grown on CMA had different metabolic patterns in response not only to the approach of nematodes, but also to the presence of living or dead nematodes. In summary, our morphological and metabolic analyses indicate complex relationships between media, fungal sensitivity, and morphological transitions. When A. oligospora was grown on CMA, it made quicker and stronger responses in both morphology and metabolism even before having direct contact with the nematodes.

The metabolomics analyses suggest a role of particular volatile metabolites in initiating the morphological transition of the nematode-trapping fungus. Volatile compounds are emitted by many species of fungi and serve many ecological functions in nature, as well as having been exploited for their role in food flavor and as indirect indicators of the presence of fungal growth (40, 41, 42). However, the role of individual fungal volatile substances in fungal-nematode ecological interactions is poorly understood.

Metabolites 1-14 were screened out from the metabolic profiles of the fungal strains grown on CMA and PDA during the switch from the saprophytic to pathogenic stages. Among them, two furanone metabolites (5 and 6) emitted by the fungus grown on CMA were found to attract the
nematodes to the fungal colony. An early study on comparison of the interaction of the free-living nematode *Panagrellus redivivus* with nematophagous fungi and non-nematophagous fungi showed that nematophagous fungi preferred to attract nematodes while non-nematophagous fungi repelled them (28). Our data not only confirm that the nematode-trapping fungi can chemically lure the prey to traps but also provides the identification of specific attractive compounds as volatile in nature.

Among the changing metabolites produced by the fungus grown on the PDA between saprotrophic and pathogenic phases, one volatile furanone metabolite, 5-methylfuran-2-carbaldehyde (8), was found to significantly paralyze and kill the nematodes. There is a long standing assumption that an unstable nematode-inactivating chemical compound produced by the fungus might be a volatile metabolite (21) and our work confirms this assumption. We also found that the amount of the long chain metabolite, (9Z, 12Z)-methyl octadeca-9,12-dienoate (14, methyl ester of linoleic acid) increased significantly in the fungus grown on PDA under contact with nematodes. Other research had identified linoleic acid as a nematicidal metabolite in the mycelial extracts of several pathogenic fungi of the genus *Arthrobotrys* (22). However, in our study, linoleic acid and its ethyl ester did not show obvious inhibitory effects on nematodes.

In addition to the furanone metabolites involved in the interaction between the fungus and nematodes, a volatile pyrone metabolite, 3-hydroxy-2-methyl-4-pyrone (maltol, 10) was identified as a morphological regulator. Maltol (10) is found widely in various beans and other plant sources such as larch tree bark, pine needles, and roasted malt (from which it gets its name) (43), but it has rarely been described as a microbial metabolite (44, 45). Maltol is responsible for much of the characteristic smell of red ginseng (46), and has been used to impart a sweet aroma to commercial fragrances. Maltol has been marketed as a safe and reliable food flavor-enhancing agent for freshly baked breads and cakes and also as food preservative and natural antioxidant (47). Maltol also is used as a bidentate metal ligand for administered drugs (45). A recent study revealed that maltol found in the root exudates from crabgrass can affect the growth of maize shoots and reduce the soil microbial biomass carbon by acting as an allelochemical that interferes with plant growth and the microbial community of soils (48). However,
our study indicates that maltol acts as a morphological regulator for fungi. In our study, maltol was found to be involved in regulating the formation of 3D traps of nematode-trapping fungus. Since maltol is widely distributed in plants, it is interesting to speculate whether there is a co-evolutionary relationship between maltol from plants attacked by the nematodes, and the induction of trap formation by nematode-trapping fungi.

Furanone and pyrone metabolites are known to be important plant fruit constituents (49, 50). For example, the 4-hydroxy-3(2H)-furanones associated with fruit aromas act to attract animals to fruits in order to ensure seed dispersal. Furanones may function as inter-organism signal molecules in various plant ecosystems (51). In plants, furanone and pyrone metabolites originate directly from carbohydrates hexoses and pentoses as Maillard reaction products (52). Previous studies have suggested that furanone might be derived from phosphorylated carbohydrates in tomato and yeast, and furaneol was from D-fructose-1,6-diphosphate. Hexose diphosphate was also assumed as biogenetic precursor to 4-hydroxy-5-methyl-2-methylene-3(2H)-furanone likely converted by an as yet unknown enzyme in tomato (Solanum lycopersicum) and strawberry (Fragaria ananassa) (43, 45). However, the biogenetic pathways of furanones and those of pyrones such as maltol (10) still remain unknown.

Polyketides are the most abundant class of fungal secondary metabolites (16). Because polyketides, furanones, and pyrone metabolites all derive from the same precursors that are obtained from hexose utilization, we studied the effects of all the PKS genes on the production of furanones and pyrone metabolites in A. oligospora. Our previous report revealed that the knockout of the PKS I-3 gene AOL_s00215g283 led to the abolishment of the morphological regulatory arthrosporals and high trap formations (30). To elucidate the effects of genes in the production of furanone and pyrone metabolites, mutants deficient in each of all the five PKS genes of A. oligospora were constructed. The mutant with loss of the PKS I-2 gene AOL_s00079g496 showed increased production of the furanone and pyrone metabolites on both CMA and PDA medium, with no obvious changes in other metabolites or on morphology. The content of nematode-attracting furanone and pyrone metabolites in the mutant strain was double that of the wildtype strain. The fact that mutant ∆AOL_s00079g496 (PKS I-2) displayed
150% stronger nematode-attracting activity was in good agreement with 200% higher of attractants in the mutant strain. Although there were fewer traps induced by the strain in which the PKS I-2 gene AOL_s00079g496 had been knocked out, the overall predatory ability remained the same as wild type. We hypothesize that the extra production of nematodetoxic furanone metabolites compensated for the lower number of traps.

In conclusion, we found that *A. oligospora* on CMA and PDA can sense the approaching nematodes and develop hyphal fusions (Fig. 10). *A. oligospora* grown on both CMA and PDA produced small volatile furanone and pyrone metabolites in response to the presence of nematodes. The fungus cultivated on CMA medium made furanone metabolites that attracted nematodes, while the fungus grown on PDA medium produced nematodetoxic furanone metabolites (Fig. 10). Mutation resulting in the increase of furanone and pyrone metabolites led to increased attractive activity and decreased trap formations of *A. oligospora* mutant, confirming the above results from integrated morphological and metabolic analysis. These results show that the fungus flexibly adjusts its metabolic activity to complement morphological changes, thereby potentially affecting fungal nematode-trapping ability and differential trap formation.

ACKNOWLEDGMENT

This work was sponsored by projects from U1502262 and 31470169, and Yunnan University Program for Excellent Young Talents awarded to X.N. (XT412003).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

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Figure Legends

Figure 1. The morphological responses of *A. oligospora* grown on two different media, CMA (red) and PDA (black), to living nematodes (solid, L) or dead nematodes (hollow, D), under two different modes of contact, direct contact (full line, DC, A) and non-direct contact (dash line, NDC, B) in 144h. 3D traps (triangle) and hyphal fusions (circle). The corrected values are the difference values between the data obtained in fungal strains treated with nematodes and those obtained in fungal strains treated without nematodes.

Figure 2. A): PCA score-plots between fungal samples during the saprophytic and pathogenic phases grown on two different media, CMA and PDA. (principal component 1 versus principal component 2; component 1, 0.85 and component 2, 0.05). B): PCA score-plots between fungal samples during the saprophytic and pathogenic phases grown on two different media CMA and PDA. (principal component 1 versus principal component 3; component 1: 0.85, component 2: 0.05 and component 3: 0.03); C): PCA score-plot between the saprophytic and pathogenic fungal samples grown on CMA (principal component 2 versus principal component 3; component 1: 0.60, component 2:0.12 and component 3: 0.07). D): PCA score-plot between the saprophytic and pathogenic fungal samples grown on PDA (principal component 2 versus principal component 3; component 1: 0.65, component 2: 0.11 and component 3: 0.08). *A. oligospora* growing without nematodes in 144h as control group (C); *A. oligospora* growing under direct contact with living nematodes in 144h (DC); *A. oligospora* growing under non-direct contact live nematodes in 144h (NDC-L); *A. oligospora* growing under non-direct contact with dead nematodes in 144h (NDC-D).

Figure 3. Unsupervised hierarchical clustering of the logarithmically transformed (log2) relative concentrations of metabolites from the methanol extracts of *A. oligospora* YMF1.01883 at different growth phases cultivated in CMA and PDA media. Up): Unsupervised hierarchical clustering of the
logarithmically transformed (log2) relative concentrations of 33 metabolites from the methanol extracts of wild type *A. oligospora* YMF1.01883 cultivated in CMA medium. Down): Unsupervised hierarchical clustering of the logarithmically transformed into (log2) metabolite relative concentrations of 16 metabolites from the methanol extracts of wild type *A. oligospora* cultivated in PDA medium. *A. oligospora* growing without nematodes in 144 h as control group (C); *A. oligospora* growing under direct contact with nematodes in 144 h (DC); *A. oligospora* growing under non-direct contact with living nematodes in 144 h (NDC-L); *A. oligospora* growing under non-direct contact with dead nematodes in 144 h (NDC-D).

Figure 4. The structures of 14 metabolites and their abundances within the time courses from the saprophytic to the pathogenic lifestyles of the fungus. (6 metabolites in the CMA group including 1-6, and 8 metabolites in the PDA group including 7-14. Control (green): *A. oligospora* growing without nematodes in 144h as control group; Direct Contact (blue): *A. oligospora* growing under direct contact with living nematodes in 144h; Non-Direct Contact Live (rose): *A. oligospora* growing under non-direct contact with living nematodes in 144h; Non-Direct Contact Dead (red): *A. oligospora* growing under non-direct contact with dead nematodes in 144h. (n=5).

Figure 5. A): Attracting activities of three metabolites, 2(5H)-furanone (5), furan-2-ylmethanol (6), and furan-2-carbaldehyde (7) for nematode *C. elegans*. B): Effect of 5-methylfuran-2-carbaldehyde (8) on the mortality of *C. elegans* at 12 hours with 1μg/mL Ivermectin used as a positive control group. C): Effect of maltol (10, M) at the concentration of 2.5 μg/mL on trap formations of *A. oligospora* as treated without maltol as control (C). **: P <0.01, *: P <0.05, n=5.

Figure 6. HPLC analysis of the methanol extracts of the PD cultural broths from the wildtype strain (black line) and the mutant ΔAOL_s00079g496 (Δ79g496) (red line). The blue line indicates the peak of 2(5H)-furanone (5) at around 4 min.
Figure 7. A): Comparison of attracting activities of wild type A. oligospora and mutant ΔAOL_s00079g496 (Δ496). B): Comparison of spore formations of A. oligospora and mutant ΔAOL_s00079g496. C): Comparison of spore germination rates of A. oligospora and mutant ΔAOL_s00079g496. D): Comparison of trap formations of wild type A. oligospora and mutant ΔAOL_s00079g496. E): Comparison of nematode capturing abilities of wild type A. oligospora and mutant ΔAOL_s00079g496. ***: P <0.001, **: P <0.01, *: P <0.05, n=4.

Figure 8. Comparison of mycelial morphology of wild-type strain and the mutant ΔAOL_s00079g496 (Δ79g496-KS) on PDA plates (15 d).

Figure 9. GC-MS analysis of the methanol extracts of the wildtype strain (black line) and the mutant ΔAOL_s00079g496 (Δ79g496) (red line) on CMA. The red arrow refers to the compounds detected in the mutant strain.

Figure 10. The morphological and metabolic adaptation of wild type A. oligospora on two media CMA and PDA. In direct contact with nematodes, the fungus grown on CMA develops more traps than those grown on PDA; in non-direct contact with nematodes, the fungus grown on PDA develops more hyphal fusion than on CMA. The fungus grown on CMA produced an attractant molecule, 2(5H)-furonone, while the fungus on PDA produced a nematicide metabolite, 5-methyl furan 2-carbaldehyde, as well as a stimulator, maltol, that increased trap numbers.
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Propanoic acid (1)  3-Ethoxy-1,2-propanediol (2)

6-Methoxy-9H-purin-2-amine (3)  Hexahydro-2,6-epoxyfuro[3,2-b]furan-3-ol (4)

2(5H)-Furanone (5)  Furan-2-ylmethanol (6)

Furan-2-carbaldehyde (7)  5-Methylfuran-2-carbaldehyde (8)

2H-Pyrane-2,6-(3H)-dione (9)  3-Hydroxy-2-methyl-4H-pyran-4-one or maltol (10)

(R)-1-phenyl-1,2-ethanediol (11)  D-(-)-Talose (12)

n-Hexadecanoic acid or palmitic acid (13)

(9Z,12Z)-Methyl octadeca-9,12-dioate (14)
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