Sivagnanapazham *et al.*
Egyptian Journal of Biological Pest Control (2022) 32:132
https://doi.org/10.1186/s41938-022-00628-1

Egyptian Journal of Biological Pest Control

RESEARCH

Open Access

Biological control of cabbage head rot (*Sclerotinia sclerotiorum*) by *Coniothyrium minitans*

K. Sivagnanapazham[1*], M. Karthikeyan[1], T. Raguchander[1], R. Swarna Priya[2] and A. Kamalakannan[1]

Abstract

Background: *Sclerotinia sclerotiorum* is the causal agent of white mould, stem and head rot diseases on a wide variety, including cabbage in the field and storage. Control of this pathogen by using commercial disease management methods is extremely difficult. Therefore, this study was performed to identify the potential biological control agent.

Results: The antagonist fungal isolates, a mycoparasite of *S. sclerotiorum*, were isolated and described as KET Cm1, PAL Cm 2 and TDK Cm 3. They were subjected to morphological and microscopic observation. The growth of antagonist fungal isolates on different media revealed that PDA and half-strength Czapek dox agar medium showed the maximum mycelial growth (28.67 and 28.33 mm) and pycnidial production in the PDA medium. The studies on pH and temperature found that growth and sporulation of the antagonist fungal isolates were optimum at pH 5.5 (34 mm) and 20 °C. The molecular confirmation of antagonist fungal isolates was carried out using ITS 1 and ITS 4 primers, which target 18S-28S rRNA gene fragment. The isolates were partially sequenced and showed that more than 99% identity with *Coniothyrium minitans* already exist in the NCBI database. The accession numbers (MW093134, MW111282 and MW136938) were obtained for all the three isolates and also confirmed using specific primers Cm sp 1F and Cm sp 1R. Among the tested isolates, the antagonistic activity of *C. minitans* TDK Cm 3 showed the maximum mycelial growth inhibition of 48.8% *S. sclerotiorum* and was used for further studies. Penetration and formation of appressoria on the hyphae of *S. sclerotiorum* by *C. minitans* isolate TDK Cm 3 were observed through the scanning electron microscope. The ethyl acetate fractions of TDK CM 3 isolate were evaluated against *S. sclerotiorum* by agar well diffusion assay, paper disc assay and poison food technique in vitro. Through GC–MS analysis, the effective antifungal compounds were identified.

Conclusion: The present study focused on the identification and characterization of effective *Coniothyrium* isolates as eco-friendly, integrated disease management strategies against cabbage head rot disease.

Keywords: *Sclerotinia sclerotiorum*, Media, pH, Temperature, Dual plate technique, SEM, GC–MS

Background

In general, control of the soil-borne diseases is difficult. Continuous use of fungicides leads to the development of resistance among the pathogen. Soil application of fungicides may imbalance microbial communities in the soil and also suppress the beneficial soil microflora (Sankar and Jeyarajan 1996). Indiscriminate use of fungicides leads to the environment and human health hazards. Biological control is the alternate and sustainable research for the control of plant diseases. Under field conditions, sclerotia are attacked and degraded by a number of mycoparasites such as *Coniothyrium minitans* and *Sporidesmium sclerotivorum* and they have been proposed as biocontrol agents for *Sclerotinia sclerotiorum* (Lib.) de Bary (Adams and Ayers 1981). *C. minitans* was

*Correspondence: sksiva1912@gmail.com

[1] Department of Plant Pathology, TNAU, Coimbatore 641003, India
Full list of author information is available at the end of the article

discovered from sclerotia in harvested samples, suggesting that this mycoparasite has the potential in reducing the survival of sclerotia in the field (Huang et al. 2000). *C. minitans* and *Gliocladium virens* have shown a practical potential for biological control of *S. sclerotiorum* under field conditions (Budge et al. 1995).

C. minitans (*Paraconiothyrium minitans*) was firstly described for biological control of *S. sclerotiorum* in California (Campbell 1947). It is a sclerotial mycoparasite and is isolated from sclerotia in soil (Sandys-Winsch et al. 1993). It has a good ability to degrade the sclerotia in soil and has a potential to control *S. sclerotiorum* by decreasing carpogenic germination and viability of sclerotia (Jones and Whipps 2002). The mode of action of *C. minitans* includes mycoparasitism, production of antibiotics and secondary metabolites, competition for space, nutrients and induction of defence responses including systemic resistance responses in the plant and reduction in the viability of carpogenic germination of sclerotia. Currently, biological control is getting great attention due to eco-friendly management and low-cost technology.

In the present study, an attempt was made to isolate and characterize the antagonist activity of *C. minitans* from the *S. sclerotiorum* sclerotia against cabbage head rot diseases.

Methods

Isolation of *Coniothyrium minitans*

Coniothyrium minitans was isolated from baiting the sclerotia in the autoclaved sand (Sandys-Winsch et al. 1993). The mycelial suspensions of *S. sclerotiorum* were sprayed over the surface-sterilized sclerotia collected from the white mould-infected cabbage, because the antagonist was found as mycoparasite in the sclerotia of *S. sclerotiorum*. Then, the sclerotia was placed in Petri plates containing 10 g of autoclaved sand. The plates were incubated for 40–45 days at 20 °C. In order to maintain the adequate humidity, 8 ml of sterile distilled water was poured into the Petri plates at weekly intervals. Some of the sclerotia showed the minute growth of fungal mycelium along with pycnidial production on the sclerotial surface (Campbell 1947). The mycelium along with pycnidia were grown on the PDA medium and assumed to be *C. minitans* (*Phaeosphaeria minitans*). The isolated *C. minitans* isolates were subjected to morphological and molecular identification.

Standardization of cultural media and temperature for the growth of *Coniothyrium* sp.

Eight culture media, viz. Potato dextrose agar, Oat meal agar, Carrot dextrose agar, Malt extract agar, Czapek dox agar, half-strength Czapek dox agar, Water agar and V8 Juice agar, were selected to identify the best media and temperature like 20 °C and room temperature (28 ± 2 °C) for growth of the *Coniothyrium* sp. All the media were prepared and sterilized at 15 psi for 30 min, and a 20 ml of sterilized warm medium was poured in sterilized Petri dishes and allowed for solidification. A 5-mm-diameter culture disc of 15-day-old culture of *Coniothyrium* sp. was placed at the centre of the plate and incubated at both 20 °C and room temperature (28 ± 2 °C). Three replications were maintained for each medium and radial growth of the fungus was measured at 10-day intervals up to 60 days after inoculation.

Effect of pH on the growth of *Coniothyrium* sp.

Sterilized PDA medium was distributed to 250-ml Erlenmeyer flasks at the rate of 100 ml per flask and the pH of the medium was adjusted to levels of 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5 with 0.1 N HCl or 0.1 N NaOH and autoclaved at 1.4 kg cm^{-2} for 20 min. Fifteen ml of the medium from each pH level was poured onto sterilized Petri dishes and allowed to solidify. A five mm culture disc of actively growing mycelium was placed at the centre of each Petri plate under an aseptic condition. The plates were incubated at 20 °C to observe the mycelial growth of the biocontrol agent.

Cultural and morphological characterization of *Coniothyrium minitans*

The isolates of *Coniothyrium minitans* were grown on Potato dextrose agar, Oat meal agar, Carrot dextrose agar, Malt extract agar, Czapek dox agar, half-strength Czapek Dox agar, Water agar, V8 Juice agar medium to study the cultural, morphological and pycnidial characters. From the 15-day-old culture plate, a 5-mm mycelial disc of fungus was cut by a sterilized cork borer and placed at the centre of each sterile Petri dish containing 20 ml of sterilized and solidified medium. The plates were incubated at 20 °C for 30 days. *C. minitans* mycelia, pycnidia and pycnidiospores characteristics were studied.

Molecular characterization of *Coniothyrium* sp. using ITS 1 and ITS 4 primers

The 20-day-old culture of *Coniothyrium* sp. was transferred into 250-ml Erlenmeyer flasks containing 150 ml PDB and incubated at 20 °C for 20 days. The mycelial mat was harvested and stored at -70° C. DNA was extracted by using CTAB buffer as described by Zhang et al. (1996). The extracted DNA was stored at -20 °C. DNA concentrations were quantified by using NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE). The fungal DNA was amplified using ITS 1 and ITS 4 Primers (sequence: 5′-TCCG ATGG TGAA CCT GCGG-3′) and ITS4 (sequence: 5′-TCCT CCGC TTATT GATA TGC-3′) according to White et al. (1990). The gradient

PCR (Eppendorf Germany) was performed with 10 µl reactions. (5 µl Master mix (Bangalore Genei Ltd., India), 1 µl ITS1 and 1 µl ITS4 primers, 2 µl double sterile distilled water, 1 µl DNA (DNA Nanodrop value—843.9 ng/µl of raw DNA). The cyclic conditions were as follows: an initial step at 95 °C for 10 min; denaturation at 94 °C for 30 s; annealing at 52 °C for 1 min followed by 35 cycles; extension at 72 °C for 1 min; final extension at 72 °C for 10 min; and hold at 10 °C (White et al. 1990). A negative control was maintained using water instead of DNA. The PCR products were examined by 1% agarose gel electrophoresis using ethidium bromide staining and documented for amplification of appropriate 560 base pairs (Sambrook 1989). For identification, the PCR products were eluted using the QIA quick gel extraction kit (Qiagen, Inc., Chatsworth, California) and sequenced using the primer ITS1 and ITS4 on an ABI 3730 XL sequencer at Chromos Biotech Pvt. Ltd., Bangalore, India. The partial sequence of the 5.8S gene and the flanking internal transcribed spacer (ITS1 and ITS4) of the isolated strains were submitted to GenBank. The amplified PCR products were sequenced by Sanger's dideoxy sequencing method and sequences were edited, aligned and the accession numbers of sequences were obtained.

Sequencing and phylogenetic tree construction

In order to study the variability, the sequences of three *C. minitans* isolates were compared with the other isolate *P. minitans* JX 496017 available in the NCBI database. The sequence identity matrix was constructed among the isolates with bio edit software version 7.2 (Kumar et al. 2016). Further, the phylogenetic tree was developed by using Mega 7.0 software including the sequence of *P. minitans* JX 496017 by maximum neighbour-joining method.

Molecular characterization of *Coniothyrium* sp. using specific primers

The ribosomal RNA gene of *C. minitans* was amplified by using specific primers Cm sp 1F (5′-CCCCAG GTGGTAA GGTGAAA-3′) and Cm sp 1R (5′-TACTAG AT GCAAAAAAGGTTTATCAG-3′) (Thompson et al. 1994). This was the species-specific primer of *C. minitans* and does not amplify the genome of *C. sporulosum* and *C. fuckelii*. PCRs were carried out in 10 µl reactions (5 µl Master mix, 1 µl *Coniothyrium* forward and 1 µl reverse primers, 2 µl Sterile distilled water, 1 µl DNA) using thermocycler. The cyclic conditions of PCR were an initial denaturation of 95 °C for 10 min; denaturation at 94 °C for 30 s; annealing at 52 °C for 1 min followed by 35 cycles; Extension at 72 °C for 1 min; final extension at 72 °C for 10 min and hold at 10 °C (White et al. 1990). Samples (5 µl) of the PCR products were separated on the 1% agarose gel in TBE buffer (90 Mm Tris-borate, 2 mM EDTA (pH 8.3) containing 0.5 µg of ethidium bromide per ml at 75 V for 2 h. The amplified PCR products were visualized with a UV illuminator and documented with an Alpha imager (Alpha Innotech Corporation, San Leandro, California).

In vitro screening of *Coniothyrium minitans* against *S. sclerotiorum*

(i) Effect of *C. minitans* on the mycelial growth of *S. sclerotiorum* by co-inoculation method

The antagonistic effects of *C. minitans* isolates (KET Cm1, PAL Cm2, TDK Cm3) were tested against *S. sclerotiorum* by dual culture technique (Dennis and Webster 1971). A 5-mm-diameter mycelial disc of 20-day-old culture of *C. minitans* was placed 1 cm away from the edge of the Petri dish containing solidified sterilized PDA medium on one side and on another side 7-day-old culture of *S. sclerotiorum* was placed at one cm away from the edge of the Petri plate just opposite to antagonist and the plates were incubated at 20 °C. Four replications were made for each treatment, and the observation of radial growth of mycelium was recorded and expressed as percentage inhibition over control. The pathogen-alone inoculated plates served as a control.

$$\mathrm{PI} = \frac{C - T}{C} \times 100$$

where PI is the percentage inhibition over control; C is the growth of test pathogen (cm) in the absence of the antagonist strain; and T is the growth of test pathogen (cm) in the presence of the antagonist strain.

(ii) Effect of pre-inoculation of *Coniothyrium minitans* isolates on the mycelial growth of *S. sclerotiorum*

Five-mm-diameter mycelial disc of *C. minitans* (KET Cm1, PAL Cm2, TDK Cm3) isolates were placed 1 cm away from the edge of the Petri plate containing sterilized solidified PDA medium. After 10 days of *C. minitans* inoculation, a 5-mm-diameter mycelial disc of 7-day-old culture of *S. sclerotiorum* was placed 1 cm away from the edge on the other side of the Petri plate. The plates were incubated at 20 °C. Control plates were maintained with pathogen alone from the 10 DAI (Days after Inoculation). Four replications were maintained for each treatment. The mycelial growth of the *S. sclerotiorum* was measured in cm and expressed as per cent inhibition over control.

Electron microscopic studies on hyperparasitism of *C. minitans* against *S. sclerotiorum*

Field emission scanning electron microscope (FESEM, SIGMA-5) was used for assessing the hyperparasitism of *C. minitans* on the hyphae of *S. sclerotiorum.* A 15 ml of PDA medium was poured into sterilized Petri plates and allowed to solidify. A 5-mm-diameter mycelial disc of *S. sclerotiorum* was placed at one end of the Petri dish and the 5-mm mycelial disc of antagonists was placed at the opposite end. The plates were incubated at 20 °C for five days. The mycelium from the zone of interaction between pathogen and antagonist was collected and the samples were processed for FESEM analysis.

The morphometric parameters of the interaction zone were viewed using scanning electron microscopy (SEM: Quanta 250, FEI, Hillsboro, OR, USA) with a large field detector (LFD). The SEM was operated in a vacuum of 10 kV with a spot size of 3.0 and a pressure of 60 Pa. The sample images were recorded at 5000 × and 10,000 × magnifications. For the analysis of hyperparasitized region, the mycelia from the interaction areas were fixed directly in carbon stubs. The source of electrons used in the FESEM was tungsten filament, and thermionic emission was used for the detection of the samples using FESEM.

Identification and characterization of secondary metabolites of *C. minitans* by gas chromatography mass spectrometry (GC–MS)

1. Extraction of antifungal antibiotic from *C. minitans*

A conical flask containing 250 ml of potato dextrose broth was autoclaved at 121.5 °C for 20 min, and then 5-mm mycelial disc of 20-day-old culture of *C. minitans* was inoculated into the broth. The flasks were incubated at 20 °C and allowed to grow into mycelial mat. After 30 days of incubation, the broth was separated using Whatman No.1 filter paper. The cultural filtrates were extracted three times with an equal volume of ethyl acetate and incubated overnight on a shaker. The solvent fraction of ethyl acetate was separated using a separating funnel. The extract was concentrated to about 150 ml by evaporation under diminished pressure using a vacuum flask evaporator, and the resulting solvent was air-dried in the sterilized Petri plate. The metabolites were scrapped using HPLC-grade methanol.

(i) Bioassay of crude antibiotics of fungal antagonist against *S. sclerotiorum* by agar well diffusion assay

The inhibitory effect of crude extracts of *Coniothyrium* isolates on the mycelial growth of *S. sclerotiorum* was studied in vitro by using Agar well diffusion method (Dhingra and Sinclair 1985). Using sterilized cork borer, a 5-mm-diameter well was made on the Petri plates containing solidified PDA medium on all four sides of the plate by leaving 1 cm away from the periphery. The 5-mm agar from the punched area was removed by using the sterilized inoculation needle. The actively growing 7-day-old culture of *S. sclerotiorum* was placed in the centre of the Petri plate. Extracted crude metabolites from the *Coniothyrium* isolates were poured into the well at the rate of 100 µl per well and incubated at 20 °C. The area of inhibition was recorded by tracing the surface area of inhibition in tracing paper and then plotted on the graph sheet and the zone of inhibition was measured. Methanol control was maintained to assess the effect of crude metabolites. All the treatments were replicated four times.

(ii) Bioassay of crude antibiotics of fungal antagonist against *S. sclerotiorum* by poison food technique

The inhibitory effects of crude metabolites at different concentrations (0.25, 0.50, 0.75 and 1%) were evaluated on the growth of *S. sclerotiorum* by poisoned food technique (Nene and Thapliyal 1993). Each treatment was replicated four times. The PDA medium without metabolite of *C. minitans* was used as control. All the plates were incubated at 20 °C for twenty days. The mycelial growth of *S. sclerotiorum* was measured in cm and expressed as percentage reduction over control. The number of sclerotia produced in each treatment was expressed in numbers.

(iii) Bioassay of crude antibiotics of fungal antagonist against *S. sclerotiorum* by paper disc assay

The filter paper was made into a 5-mm-diameter disc by using a punching machine, and these paper discs were placed in the Petri plates and autoclaved it. The autoclaved paper discs were dipped in the metabolite suspension and placed on all four sides of the Petri plate containing sterilized solidified PDA medium and leaving one cm away from the periphery. A 5-mm mycelial disc of *S. sclerotiorum* was placed at the centre of the Petri plate and the plates were incubated at 20 °C. The paper disc dipped in water was maintained as control. Three replications were made for each treatment and the growth of mycelium and inhibition zone around the paper discs were observed and expressed in per cent reduction over control.

GC–MS analysis of crude antibiotics

The crude antibiotics of the effective isolates of *C. minitans* were analysed for the detection of active biomolecules

responsible for the suppression of *S. sclerotiorum* through GC–MS (GC Clarus 500 PerkinElmer). The volatile components were identified by GC–MS using a column Elite-5MS (100% dimethylpolysiloxane), 30 mm × 0.25 mm × 0.25 µm df equipped with GC Clarus 500 PerkinElmer. The Turbo-Mass Gold PerkinElmer detector was used. The carrier gas flow rate was 1 ml per min, split 10:1 and injected volumes were 3 µl. The column temperature was maintained initially at 110 °C at the rate of 10 °C/min-No hold was followed by increasing up to 280 °C at the rate of 5° C /min with 9 min (hold). The injector temperature was 250 °C and this temperature was held constant for 36 min. The electron impact energy was 70 eV, the Julet line temperature was set at 2000 °C, and the source temperature was set at 200 °C. Electron impact (EI) mass scan (m/z) was recorded in the 45–450 a MU range. Using computer searches on the NIST Ver.2005 MS data library and comparing the spectrum obtained through GC/MS, the compounds present in the crude sample were identified.

Results

Isolation and morphological characterization of *Coniothyrium minitans*

The biocontrol agent associated with sclerotia of the head rot pathogen was isolated. A total of three isolates were isolated and identified as *C. minitans*, based on the morphological characteristics and designated as KET Cm1, PAL Cm2 and TDK Cm3. The colony colour of *C. minitans* isolates was initially white and later changed to grey at the centre. The *Coniothyrium* isolates KET Cm1 and PAL Cm2 produced white to grey colonies, while TDK Cm3 isolate produced white colony with pale green concentric rings. The mycelia of *Coniothyrium* sp. showed a slow growth and lasted around 60 days to cover Petri plates. Under the surface of the Petri plate, it showed dark brown colour due to melanin accumulation. The pycnidia of *C. minitans* were 150–700 µm in diameter, ostiolate, globose, brown to black, sub-globose at maturity. Pycnidiospores were dark brown colour, ovoid to ellipsoid, nearly globose smooth to roughened (Tables 1, 2; Fig. 1a, b).

Molecular characterization of *Coniothyrium* sp.

Agarose gel electrophoresis amplified the genomic DNA of three *C. minitans* isolates and yielded an amplicon size of approximately 560 base pairs. The rDNA homology sequences analysis using BLAST revealed that all the three isolates share more than 99% nucleotide sequence identity with the existing *C. minitans* isolates available in the NCBI database. A nucleotide sequence of three isolates was submitted to NCBI for getting accession number. The accession numbers of three *Coniothyrium* isolates are listed in Table 3, Figs. 2 and 3.

Table 1 Isolation of *Coniothyrium* species from isolates of *Sclerotinia sclerotiorum*

S. No.	Location	District	Isolates of *S. sclerotiorum*	Presence of *Coniothyrium minitans*
1	Narasipuram	Coimbatore	CBESS1	–
2	Ketti	The Nilgiris	KETSS2	+
3	Muthorai	The Nilgiris	MTRSS3	–
4	Palada	The Nilgiris	PALSS4	+
5	Nanjanadu	The Nilgiris	NAJSS5	–
6	Kappathorai	The Nilgiris	KAPSS6	–
7	Emerald	The Nilgiris	EMRSS7	–
8	Vilpatti	Dindigul	PMBSS8	–
9	Poombarai	Dindigul	VILSS9	–
10	Thadiyankudisai	Dindigul	TDKSS10	+

(+) indicates the presence of *C. minitans*, (–) indicates the absence of *C. minitans*

The identification of fungus *C. minitans* was further confirmed by molecular characterization using ITS and species-specific primers. In the present study, PCR and agarose gel electrophoretic reaction yielded an amplicon size of approximately 560 bp corresponding to ITS region. The fungus was further confirmed through species level through PCR with a specific primer, and an amplicon size of 170 bp size was recorded. The amplified products of ITS and species-specific primers were partially sequenced and identified as *Coniothyrium minitans* by comparing sequences available in the NCBI database. Species-level confirmation of *C. minitans* isolates was performed by PCR assay using species-specific primer Cm sp1F and Cm sp 1R. The genomic DNA of three isolates of *C. minitans* yielded approximately 170 bp size of amplicon in agarose gel electrophoresis assay. This has confirmed that all the three *Coniothyrium* isolates belonged to *C. minitans* (Fig. 3).

Phylogenetic analysis of *Coniothyrium minitans* isolates

The phylogenetic analysis was carried out using MEGA7.0 software in order to analyse the genetic diversity among the other isolates of *Coniothyrium.* From this study, the result revealed that the two major clads were formed. In clad 2, isolate TDK Cm3 was out-grouped from the major clad 1 which showed diverse genetic bases from other isolates. The major clad 1 was further divided into two sub-clads. In sub-clad 2, the isolate KET Cm1 was shown 100 per cent genetically similar to the isolates JX 496017 and PAL Cm2 which was clustered together as sub-clad 1(Fig. 4).

Table 2 Morphological characterization of *Coniothyrium minitans* in different media

S. No.	Media	Colony growth	Growth pattern	Colony colour	Growth rate (mm/day)	Pycnidial production	Days took for Pycnidia production
1	PDA	Moderate	Flat	White to pale green	0.6	+	20
2	MEA	Fast	Flat	Pale white	0.63	–	–
3	CZA	Slow	Constricted	Whitish brown	0.4	–	–
4	HCZA	Rapid	Flat	Whitish brown	0.56	+	23
5	WA	Slow	Sparse	Black	0.54	–	–
6	OMA	Moderate	Radial	Grey	0.65	+	30
7	CDA	Slow	Flat	White to pale brown	0.25	–	–
8	V8	Slow	Flat	Yellow	0.47	–	–

(+) indicates the production of pycnidia in the medium, (–) indicates the absence of pycnidial production

The phylogenetic analysis of *C. minitans* revealed that the two major clads were formed. In clad 2, isolate TDK Cm3 was out grouped from the major clad 1 which showed diverse genetic bases from other isolates. The major clad 1 was further divided to two sub-clads. In sub-clad 2, the isolate KET Cm 1 was shown 100% genetically similar to the existing isolate JX 496017 available in GenBank and PAL Cm 2 which was clustered together as sub-clad 1.

Standardization of culture media and temperature for the growth of *Coniothyrium minitans*

All the culture media evaluated for the growth of *C. minitans* supported the mycelial growth at 20 °C and room temperature 28 ± 2 °C. Among two temperatures tested, 20 °C supported the mycelial growth of *C. minitans* more than room temperature. At 20 °C PDA recorded the maximum mycelial growth (28.67 mm), followed by H.CZA (28.33 mm), WA (26.67 mm), V8 juice agar (25.67 mm), OMA (24.17 mm) and MEA (24.83 mm). The lowest mycelial growth of 8.45 mm was observed in the CDA medium. At room temperature (28 ± 2 °C), the maximum mycelial growth was observed in PDA (26.33 mm), followed by V8 juice agar (24.33 mm), CDA (24.00 mm), OMA (19.95 mm), MEA (19.16 mm) and H.CZA (18.48 mm). The lowest mycelial growth of 10.68 mm was recorded in WA (Tables 4 and 5; Figs. 5a–h and 6 a–h).

Standardization of pH for the growth of *Coniothyrium minitans*

The effect of different pH levels of PDA medium on the mycelial growth of *C. minitans* was tested with a range from 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5, and the results are presented in Table 6. The results revealed that among the different pH levels tested, pH 5.5 exerted maximum mycelial growth of 34 mm. This was followed by pH 6.5 (33 mm), pH 6.0 (32 mm), pH 5.0 (27 mm) and pH 4.5 (23 mm). The lowest pH 4.0 recorded the least mycelial growth of 14 mm (Figs. 7, 8). The pH of cultural media had also influenced the hyphal extension and pycnidial formation in *C. minitans*. Though hyphal extension was observed in different pH, cultural medium with 5.5 pH recorded maximum mycelial growth.

In vitro screening of *Coniothyrium minitans* against *S. sclerotiorum*

1. Co-inoculation

The effect of concurrent inoculation of *C. minitans* and *S. sclerotiorum* was tested in vitro using the dual plate method (Table 7). Among the three *Coniothyrium* isolates tested, the *C. minitans* TDK Cm 3 isolate showed maximum mycelial inhibition of 28.8% over control. This was followed by KET Cm1 and PAL Cm2 which recorded an inhibitory effect of 27.7% and 25.5% reduction over control, respectively (Fig. 9).

2. Pre-inoculation

The effect of *C. minitans* against the mycelial growth of *S. sclerotiorum* was also studied by pre-inoculation of the former one. The results revealed that the maximum inhibition of mycelial growth was recorded at 48.8% in pre-inoculated *Coniothyrium* TDK Cm3 isolate over control. The isolate KET Cm1 was on par with TDK Cm3 in reducing the mycelial growth of *S. sclerotiorum*. The least mycelial growth inhibition was recorded in PAL Cm2 with a 44.4% reduction over control (Table 7; Fig. 10).

SEM analysis of *C. minitans* against *S. sclerotiorum*

The hyperparasitic region of *S. sclerotiorum* was studied through SEM analysis. The results revealed that the hyphae of *S. sclerotiorum* showed abnormal swelling with

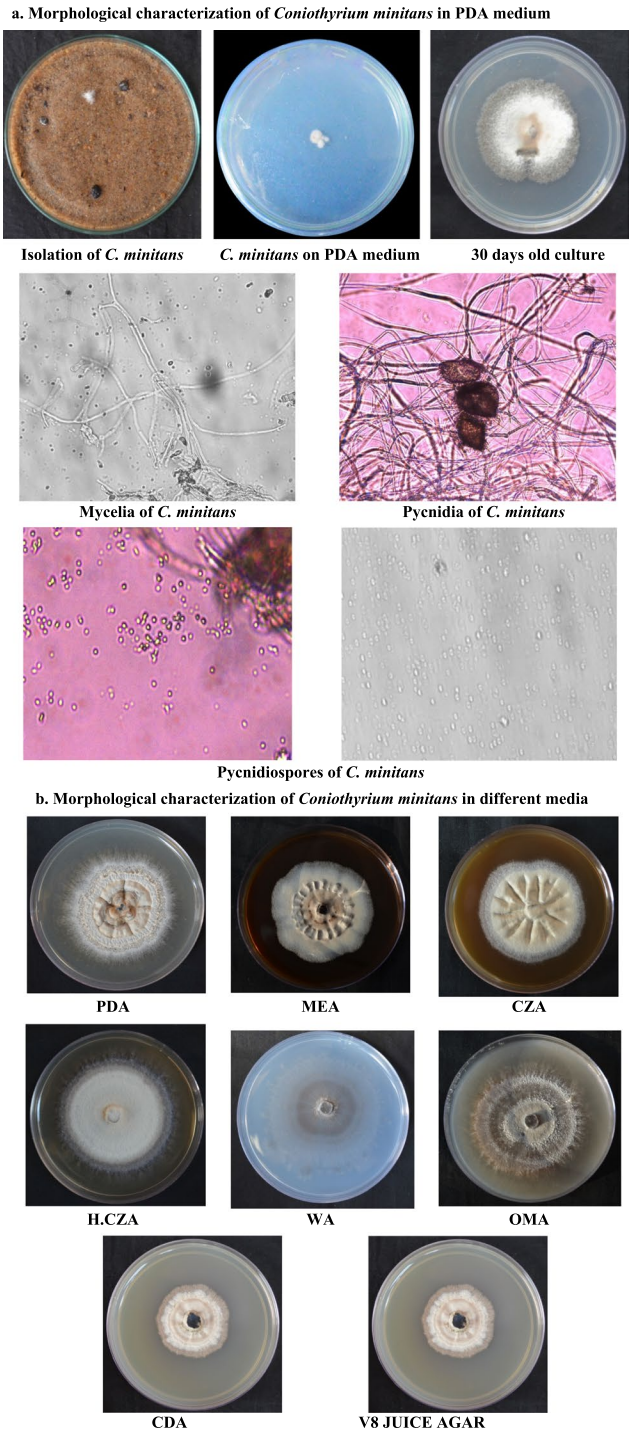
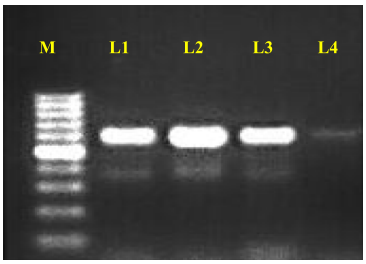


Fig. 1 **a** Morphological characterization of *Coniothyrium minitans* in the PDA medium. **b** Morphological characterization of *Coniothyrium minitans* in different media. PDA, potato dextrose agar; MEA, malt extract agar; CZA, Czapek dox agar; H.CZA, half-strength Czapek dox agar; WA, water agar; OMA, oat meal agar; CDA, carrot dextrose agar; V8, V8 juice agar

Table 3 Molecular identification of *Coniothyrium minitans* isolates

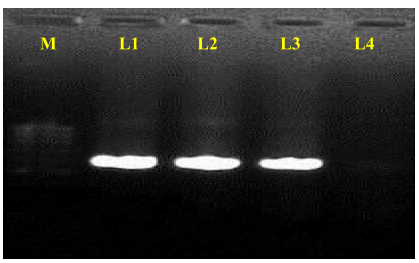
S. No.	Isolates	Sources	Species identified	Accession number	Query coverage (%)	Per cent identity
1	KET Cm 1	Ketti	*Coniothyrium minitans*	MW093134	100	100
2	PAL Cm 2	Palada	*Coniothyrium minitans*	MW111282	100	99
3	TDK Cm 3	Thadiyankudusai	*Coniothyrium minitans*	MW136938	100	99.95



PCR amplicons of ITS regions of *C. minitans*. on 1.2% agarose gel electrophoresis.

M – 100 bp DNA ladder; Lane 1, KET Cm 1; Lane 2, PAL Cm 2; Lane 3, TDK Cm 3; Lane 4, Positive control.

Fig. 2 Molecular identification of *C. minitans* isolates using ITS 1 and ITS 4 primers. PCR amplicons of ITS regions of *C. minitans* on 1.2% agarose gel electrophoresis. M – 100 bp DNA ladder; Lane 1, KET Cm 1; Lane 2, PAL Cm 2; Lane 3, TDK Cm 3; Lane 4, Positive control



PCR amplicons of ITS regions of *C. minitans*. on 1.2% agarose gel electrophoresis.

M – 100 bp DNA ladder; Lane 1, KET Cm 1; Lane 2, PAL Cm 2; Lane 3, TDK Cm 3; Lane 4, negative control.

Fig. 3 Molecular identification of *C. minitans* isolates using CM sp 1F and CM sp 1R specific primers. PCR amplicons of ITS regions of *C. minitans* on 1.2% agarose gel electrophoresis. M – 100 bp DNA ladder; Lane 1, KET Cm 1; Lane 2, PAL Cm 2; Lane 3, TDK Cm 3; Lane 4, negative control

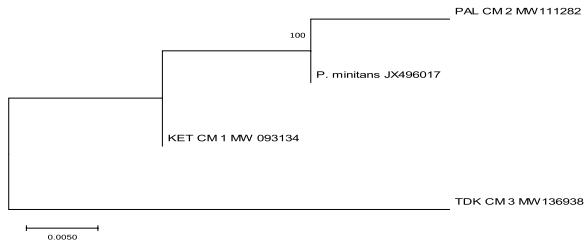


Fig. 4 Phylogenetic tree generated from a neighbour-joining (NJ) analysis using 5.8S rRNA sequence of *Coniothyrium minitans* using MEGA 7 software. Number besides branches represents the percentage of congruent cluster in 1000 bootstrap trails. The bar indicates 1 per cent sequences dissimilarity

malformations. Some parts of the hyphae became disintegrated due to the lysis action of the enzymes secreted by *C. minitans*. The hyphae of *C. minitans* formed appressoria on the surface of *S. sclerotiorum* hyphae. The above abnormalities observed in *S. sclerotiorum* hyphae and the formation of appressoria by *C. minitans* hyphae confirmed the hyperparasitic nature of *C. minitans* (Fig. 11). The FESEM analysis of the hyphae of *S. sclerotiorum* excised from the lytic zone around the *C. minitans* revealed that hyphal lysis, formation of appressoria and malformation of hypha.

Antifungal activity of secondary metabolites of *C. minitans* against *S. sclerotiorum*

1. Agar well diffusion assay

The effect of secondary metabolites secreted by *C. minitans* isolates on the mycelial growth of *S. sclerotiorum* was studied using agar well diffusion method (Table 8). The results revealed that KET Cm1 and TDK Cm3 isolate significantly reduced the mycelial growth of *S. sclerotiorum* (88.8%) than control. This was followed by isolate PAL Cm 2 which recorded mycelial growth inhibition of 83.3% reduction over control. Sterilized water control did not inhibit the mycelial growth of *S. sclerotiorum* and this recorded higher mycelial growth of 9 cm (Fig. 12).

2. Paper disc assay

The effect of crude secondary metabolites secreted by *C. minitans* isolates against mycelial growth of *S. sclerotiorum* was also studied by paper disc assay (Table 8). The results revealed that the mycelial growth of *S. sclerotiorum* was inhibited by all the three *C. minitans* isolates. The inhibition percentage ranged from 37.7 to 40.0%. Among the three different isolates, maximum inhibition

Table 4 Mycelial growth of *Coniothyrium minitans* TDK Cm3 in different media at 20 °C

S. No.	Different media	10th day (mm)*	20th day (mm)*	30th day (mm)*	40th day (mm)*	50th day (mm)*	60th day (mm)*	Mean (mm)*
1	PDA	18.0^{b}	25.0^{a}	30.0^{a}	32.0^{a}	33.0^{ab}	34.0^{c}	28.67
2	MEA	12.0^{d}	17.0^{e}	23.0^{d}	27.0^{b}	32.0^{b}	38.0^{ab}	24.83
3	CZA	1.0^{f}	15.0^{f}	18.0^{f}	20.0^{d}	25.0^{d}	30.0^{d}	18.17
4	H.CZA	15.0^{c}	24.0^{b}	30.0^{a}	32.0^{a}	33.0^{ab}	36.0^{bc}	28.33
5	WA	11.0^{e}	19.0^{d}	28.0^{b}	33.0^{a}	34.0^{a}	37.0^{ab}	26.67
6	OMA	12.0^{d}	15.0^{f}	20.0^{e}	25.0^{c}	34.0^{a}	39.0^{a}	24.17
7	CDA	0.8^{f}	0.9^{g}	10.0^{g}	12.0^{e}	12.0^{e}	15.0^{e}	8.45
8	V8	20.0^{a}	23.0^{c}	25.0^{c}	27.0^{b}	29.0^{c}	30.0^{d}	25.67
	CD	1.48	0.92	1.12	1.41	1.11	2.07	

In the column, means followed by a common letter are not significantly different at 5% level by DMRT

*Values are the means of four replications

Table 5 Mycelial growth of *Coniothyrium minitans* TDK Cm3 in different media at room temperature (28 ± 2 °C)

S. No.	Different media	10th day (mm)*	20th day (mm)*	30th day (mm)*	40th day (mm)*	50th day (mm)*	60th day (mm)*	Mean (mm)*
1	PDA	15.0^{b}	25.0^{a}	27.0^{a}	28.0^{a}	30.0^{a}	33.0^{b}	26.33
2	MEA	10.0^{d}	12.0^{f}	16.0^{g}	20.0^{c}	25.0^{c}	32.0^{b}	19.16
3	CZA	0.8^{e}	15.0^{e}	19.0^{e}	21.0^{c}	23.0^{d}	25.0^{e}	17.30
4	H.CZA	0.9^{e}	19.0^{d}	20.0^{d}	20.0^{c}	23.0^{d}	28.0^{d}	18.48
5	WA	0.2^{f}	0.9^{g}	11.0^{h}	13.0^{d}	18.0^{e}	21.0^{f}	10.68
6	OMA	0.7^{e}	12.0^{f}	17.0^{f}	25.0^{b}	30.0^{a}	35.0^{a}	19.95
7	CDA	12.0^{c}	23.0^{b}	25.0^{b}	27.0^{a}	28.0^{b}	29.0^{cd}	24.00
8	V8	20.0^{a}	22.0^{c}	23.0^{c}	24.0^{b}	27.0^{b}	30.0^{c}	24.33
	CD	0.38	1.05	0.83	1.13	1.17	1.49	

In the column, means followed by a common letter are not significantly different at 5% level by DMRT

*Values are the means of four replications

was exerted by the isolate TDK Cm 3 (40.0%) with an inhibition zone of 1 mm. The isolate KET Cm1 was on par with PAL Cm2 in reducing mycelial growth (Fig. 13).

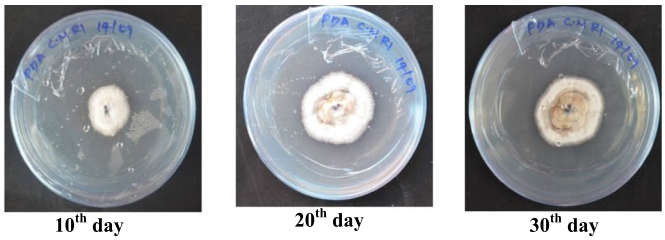
3. Poison food technique

The effect of secondary metabolites produced by the elite *C. minitans* isolates, TDK Cm3 was tested under the poison food technique (Table 9). Secondary metabolites secreted by TDK Cm3 showed an inhibitory effect on the mycelial growth of *S. sclerotiorum.* Among the four concentrations of secondary metabolite tested, low concentrations of 0.25 and 0.50% did not show any inhibitory effect on mycelial growth and sclerotial productions. High concentration of secondary metabolites like 0.75 and 1% showed a significant reduction in the mycelial growth and cent per cent inhibition of sclerotial productions. The maximum inhibition of mycelial growth was recorded in 1% concentration of crude metabolites of *C. minitans* (55%). Though less inhibition (11.1%) was

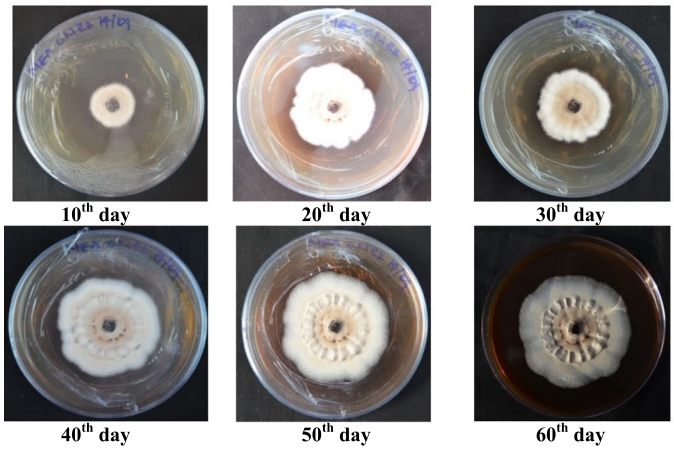
(See figure on next page.)

Fig. 5 **a** Mycelial growth of *Coniothyrium minitans* in the PDA medium at 20 °C at different days interval. **b** Mycelialgrowth of *Coniothyrium minitans* in malt extract agar medium at 20 °C at different days interval. **c** Mycelial growth of *Coniothyrium minitans* in Czapek Dox agar medium at 20 °C at different days interval. **d** Mycelial growth of *Coniothyrium minitans* in half-strength Czapek Dox agar medium at 20 °C at different days interval. **e** Mycelial growth of *Coniothyrium minitans* in water agar medium at 20 °C at different days interval. **f** Mycelial growth of *Coniothyrium minitans* in oat meal agar medium at 20 °C at different days interval. **g** Mycelial growth of *Coniothyrium minitans* in carrot dextrose agar medium at 20 °C at different days interval. **h** Mycelial growth of *Coniothyrium minitans* in V8 juice agar medium at 20 °C at different days interval

a. Mycelial growth of *Coniothyrium minitans* in PDA medium at 20°C at different days interval



b. Mycelial growth of *Coniothyrium minitans* in Malt extract agar medium at 20°C at different days interval



c. Mycelial growth of *Coniothyrium minitans* in Czapek Dox Agar medium at 20°C at different days interval

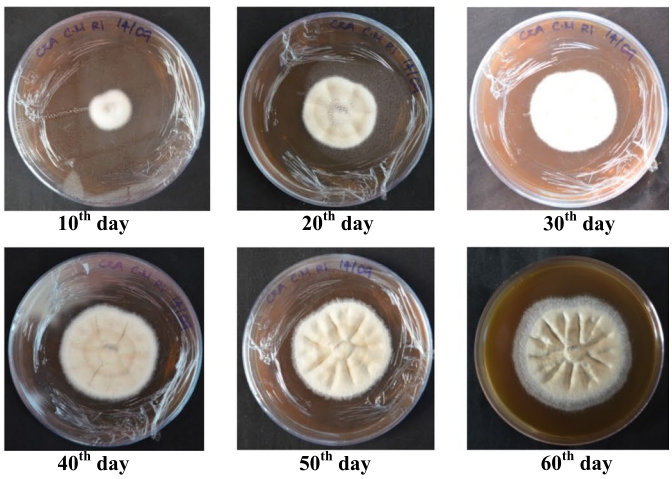
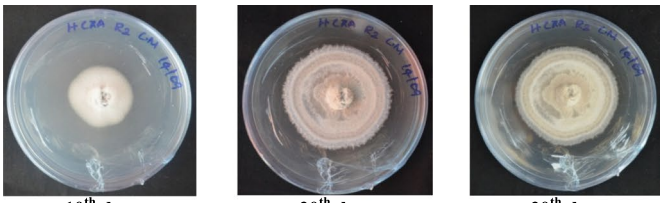
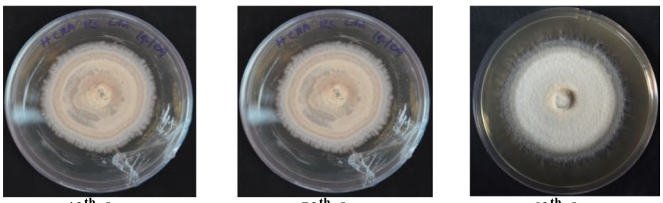


Fig. 5 (See legend on previous page.)

d. Mycelial growth of *Coniothyrium minitans* in Half strength Czapek Dox Agar medium at 20°C at different days interval

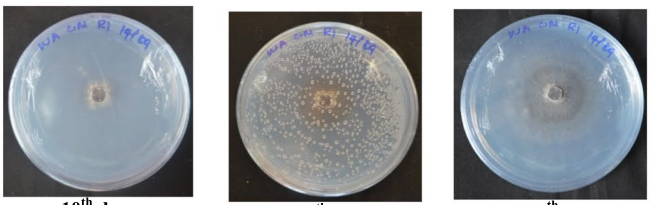


10th day 20th day 30th day

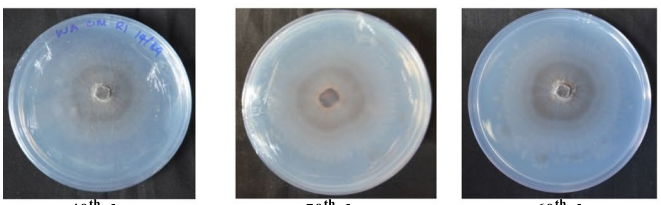


40th day 50th day 60th day

e. Mycelial growth of *Coniothyrium minitans* in Water Agar medium at 20°C at different days interval

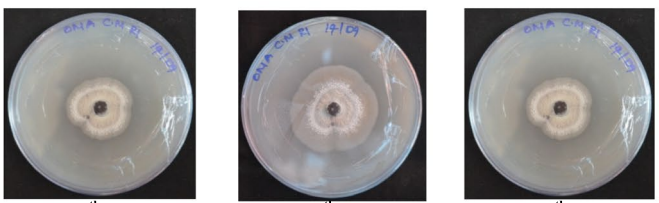


10th day 20th day 30th day

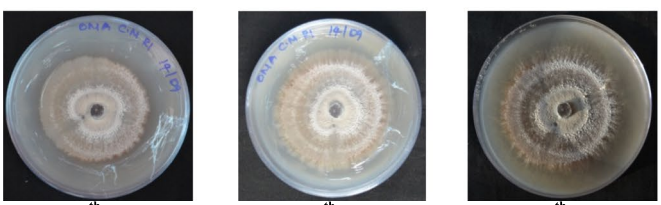


40th day 50th day 60th day

f. Mycelial growth of *Coniothyrium minitans* in Oat Meal Agar medium at 20°C at different days interval



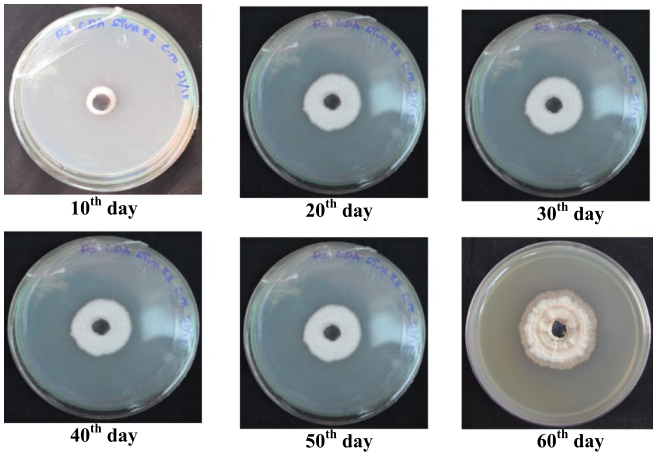
10th day 20th day 30th day



40th day 50th day 60th day

Fig. 5 continued

g. Mycelial growth of *Coniothyrium minitans* in Carrot Dextrose Agar medium at 20°C at different days interval



h. Mycelial growth of *Coniothyrium minitans* in V8 Juice Agar medium at 20°C at different days interval

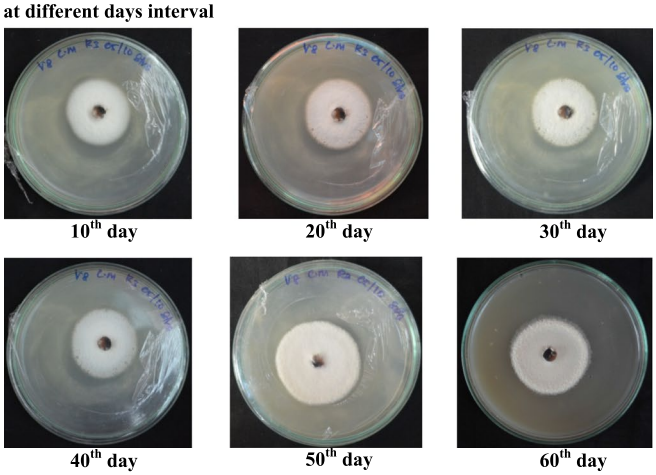


Fig. 5 continued

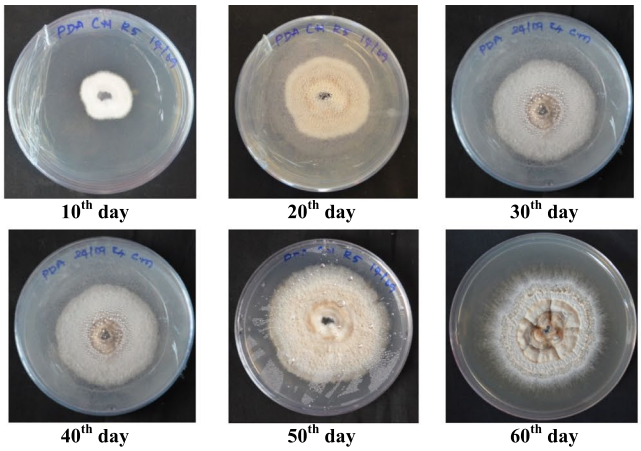
observed at 0.75% concentration, the inhibition of sclerotial production was cent % (Fig. 14).

The effect of NVOM produced by the effective isolate of *C. minitans* TDK Cm3, was analysed using agar well diffusion assay, paper disc assay and poison food technique. In all the three assays, the NVOM effectively inhibited the mycelial growth of *S. sclerotiorum*. The inhibitory effect of NVOM may be due to the antimicrobial properties of bioactive compounds present in non-volatile fraction.

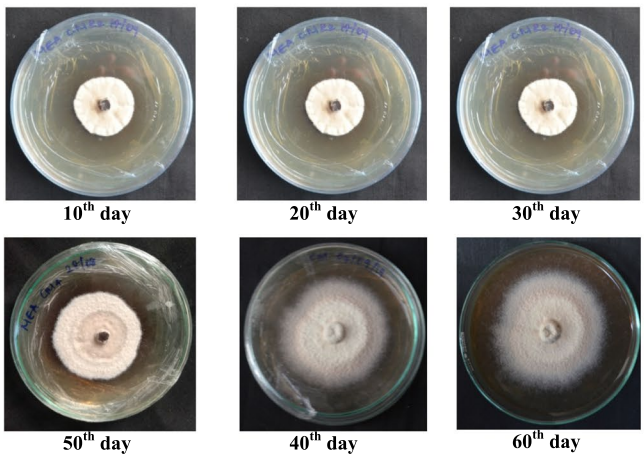
(See figure on next page.)

Fig. 6 **a** Mycelial growth of *Coniothyrium minitans* in the PDA medium at room temperature (28 ± 2 °C) at different days interval. **b** Mycelial growth of *Coniothyrium minitans* in malt extract agar medium at room temperature (28 ± 2 °C) at different days interval. **c** Mycelial growth of *Coniothyrium minitans* in Czapek Dox agar medium at room temperature (28 ± 2 °C) at different days interval. **d** Mycelial growth of *Coniothyrium minitans* in half-strength Czapek Dox agar medium at room temperature (28 ± 2 °C) at different days interval. **e** Mycelial growth of *Coniothyrium minitans* in water agar medium at room temperature (28 ± 2 °C) at different days interval. **f** Mycelial growth of *Coniothyrium minitans* in oat meal agar medium at room temperature (28 ± 2 °C) at different days interval. **g** Mycelial growth of *Coniothyrium minitans* in carrot dextrose agar medium at temperature (28 ± 2 °C) at different days interval. **h** Mycelial growth of *Coniothyrium minitans* in V8 juice agar medium at temperature (28 ± 2 °C) at different days interval

a. Mycelial growth of *Coniothyrium minitans* in PDA medium at room temperature (28±2^{0}C) at different days interval



b. Mycelial growth of *Coniothyrium minitans* in Malt extract agar medium at room temperature (28±2^{0}C) at different days interval



c. Mycelial growth of *Coniothyrium minitans* in Czapek Dox agar medium at room temperature (28±2^{0}C) at different days interval

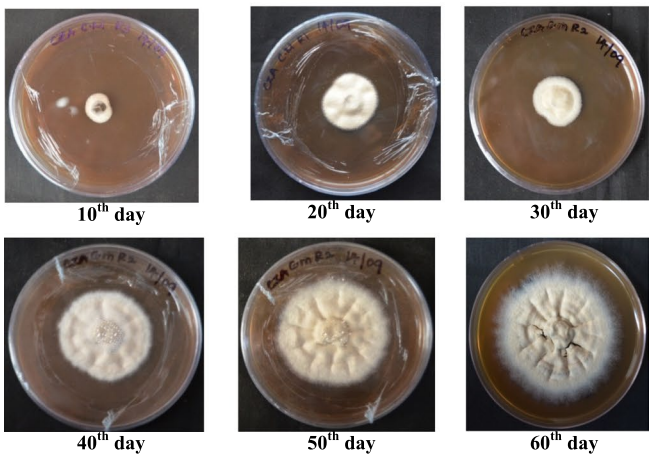
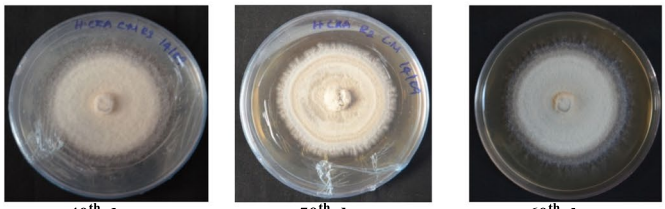


Fig. 6 (See legend on previous page.)

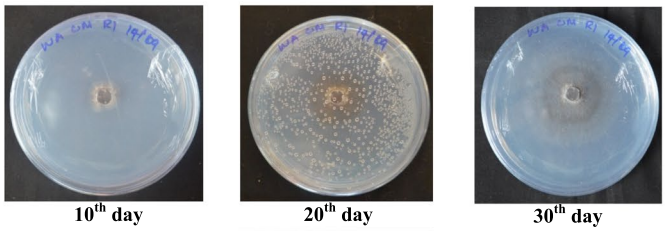
d. Mycelial growth of *Coniothyrium minitans* in Half strength Czapek Dox Agar medium at room temperature (28±2^{0}C) at different days interval

10th day 20th day 30th day

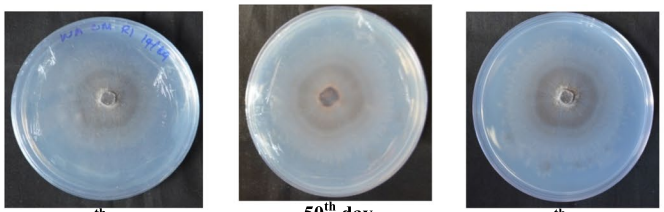


40th day 50th day 60th day

e. Mycelial growth of *Coniothyrium minitans* in Water agar medium at room temperature (28±2^{0}C) at different days interval

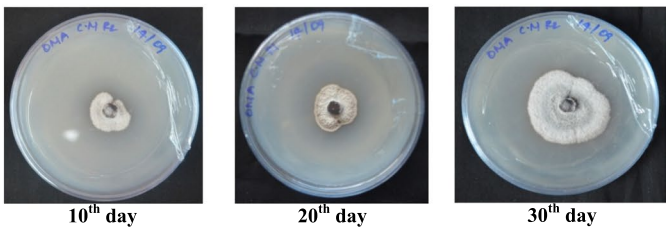


10th day 20th day 30th day

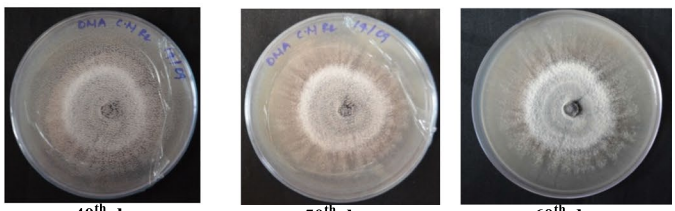


40th day 50th day 60th day

f. Mycelial growth of *Coniothyrium minitans* in Oat Meal agar medium at room temperature (28±2^{0}C) at different days interval



10th day 20th day 30th day



40th day 50th day 60th day

Fig. 6 continued

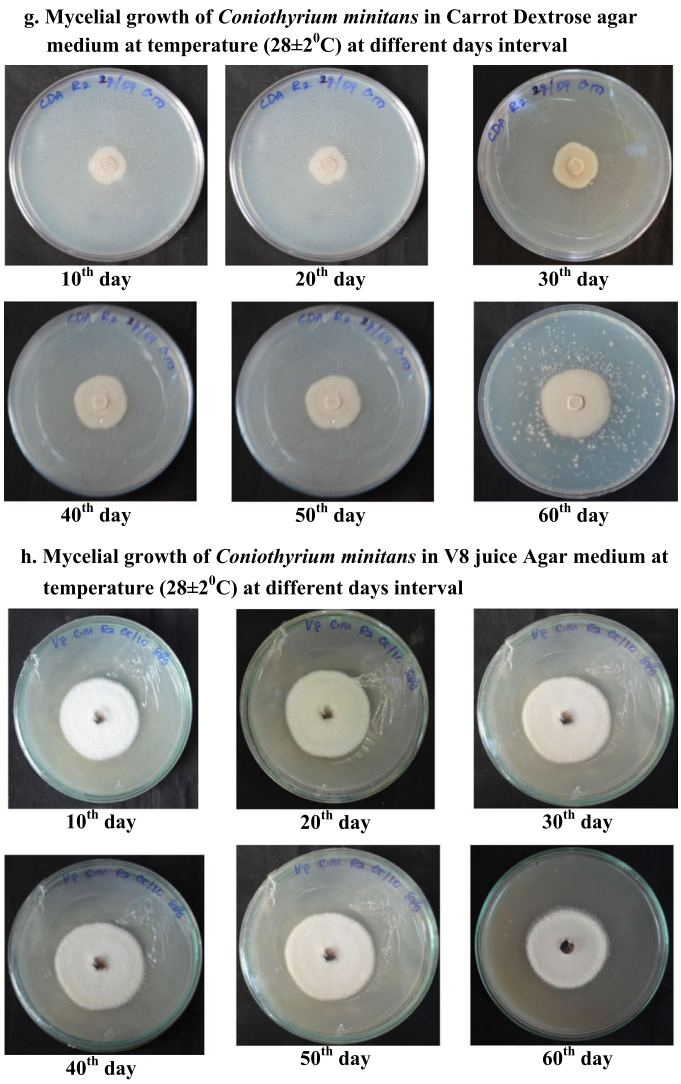


Fig. 6 continued

Table 6 Influence of different pH level of PDA on the mycelial growth of TDK Cm3 isolate

S. No.	pH	Mean (mm)*
1	4.0	14^{e}
2	4.5	23^{d}
3	5.0	27^{c}
4	5.5	34^{a}
5	6.0	33^{ab}
6	6.5	33^{ab}
	CD	1.08

In the column, means followed by a common letter are not significantly different at 5% level by DMRT

*Values are the means of three replications

Metabolic profiling of non-volatile organic compounds produced by *Coniothyrium minitans* TDK Cm3 isolate

The secondary metabolites with antimicrobial compounds produced by *C. minitans* TDK Cm 3 isolate were analysed through GCMS (Table 10). A total of 40 compounds was detected through GCMS analysis, out of these 10 compounds were selected based on relative abundance and peak area percentage. The results revealed that the secondary metabolites contained the biomolecules with antifungal activity. The biomolecules detected were decane, benzothiazole, tetradecanoic acid, 1-nonadecene, phytol acetate octadecynoic acid, dimethyl palmitamine, n-hexadecenoic acid, 1-heneicosylformate and 1-heptatriacotanol (Fig. 15).

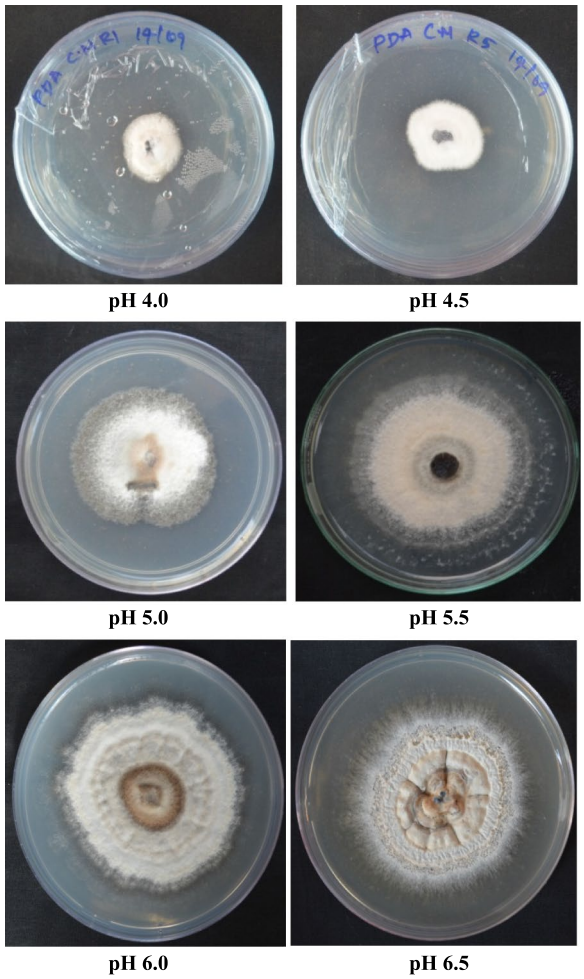


Fig. 7 Mycelial growth of *Coniothyrium minitans* TDK Cm3 in different pH levels on PDA medium

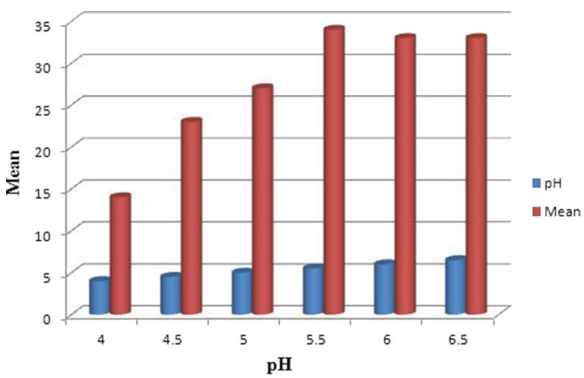


Fig. 8 Influence of different pH levels on the mycelial growth of *C. minitans* on PDA medium

The GC MS analysis of non-volatile organic compounds secreted by *C. minitans* isolate TDK Cm 3 revealed that the ethyl acetate fraction of culture filtrate contained 10 different organic compounds with antifungal, antibacterial and antioxidant activities. The antifungal compounds included decane, benzothiazole, tetradecanoic acid, 1-nonadecene, phytol acetate, octadecynoic acid, dimethyl palmitamine, n-hexadecenoic acid, 1-heneicosylformate and 1-heptatriacotanol. Apart from the seven antifungal compounds, the compounds with antibacterial activity were also recorded in GC MS analysis. These compounds with antifungal activity may be responsible for the reduction in mycelial growth under in vitro conditions.

Discussion

Coniothyrium minitans was firstly described for the biological control of *S. sclerotiorum* in California by Campbell (1947). It was isolated from the sclerotia of *S. sclerotiorum* from more than 30 countries on all continents (Sandys-Winsch et al. 1993). This fungus is ecologically fastidious mycoparasite on sclerotia of *S. sclerotiorum*, *Botrytis* spp. and *S. cepivorum.* (Wang et al. 2008). *C. minitans* displayed a good ability to infect and degrade the sclerotia present in the soil by decreasing carpogenic germination and viability of sclerotia. Ojaghian (2009) isolated *C. minitans* from sclerotia of *S. sclerotiorum* infecting potato on PDA medium. It was described that *C. minitans* was slow growing with non-aerial mycelium and produced pycnidia 3 weeks after inoculation. *C. minitans* fungal colony was initially white and became grey after two weeks, without aerial mycelium. Its hyphae were 3 to 6 µm diameter, smooth, and simple with numerous septa (Whipps 1992). The pycnidia were 150–700 µm in size, ostiolate, brown to black, sub-globose which produced pycnidiospores of dark brown, and ovoid to ellipsoid are globose pycnidiospores (Punithalingam 1982). In the present study, three isolates of *C. minitans* were isolated and characterized morphologically. The fungal colony characters, mycelial characters, pycnidial and pycnidiospores were in accordance with characters of *C. minitans* described by earlier workers. Earlier the identity of *C. minitans* was molecularly confirmed by Muthumeenakshi et al. (2001) by using ITS 1 & ITS 4 primers and specific primers. Similarly, Muthumeenakshi et al. (2001) studied the intraspecific diversity and phylogenetic position of *C. minitans.* The result of the above findings showed that two major clad were formed; from major clad 1, the sub-clad 2 includes *C. minitans* and *C. sporulosum* which were clustered together and showed 100% similarity with each isolate.

Table 7 In vitro screening of *Coniothyrium minitans* isolates against *S. sclerotiorum* NAJ SS5 at 20 °C (co-inoculation and pre-inoculation method)

S. No.	Treatments	Growth of *Sclerotinia sclerotiorum* (cm)*	Growth of *Coniothyrium minitans* (cm)*	Per cent of pathogen inhibition over control **
Co-inoculation				
1	KETCm1	6.5	1.8	27.7[b] (31.76)
2	PAL Cm2	6.7	1.8	25.5c (31.76)
3	TDKCm3	6.4	1.5	28.8[a] (32.46)
4	Control	9.0	0.0	0.0
			CD	0.70
Pre-inoculation				
1	KETCm1	4.7	3.2	47.7b (43.68)
2	PAL Cm2	5.0	3.2	44.4c (41.78)
3	TDK Cm3	4.6	3.3	48.8a (44.31)
4	Control	9.0	0.0	0.0
			CD	0.59

In the column, means followed by a common letter are not significantly different at 5% level by DMRT

*Values are the means of four replications

**Values in the parentheses are arc sign-transformed values

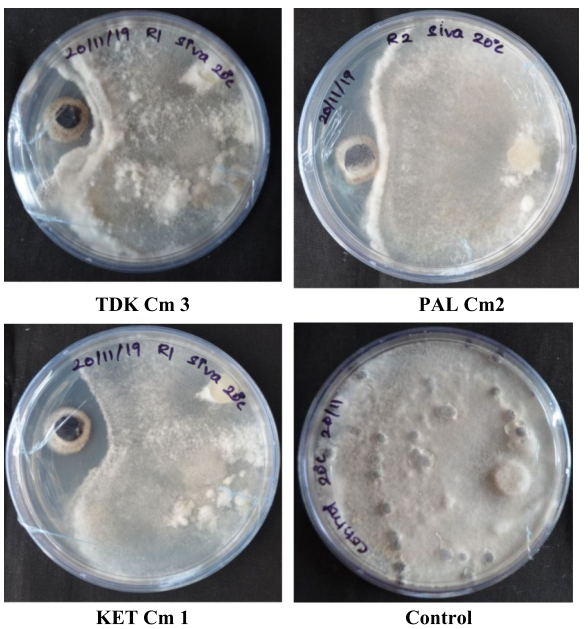


Fig. 9 In vitro screening of *Coniothyrium minitans* against *Sclerotinia sclerotiorum* NAJ SS5 at 20 °C (co-inoculation)

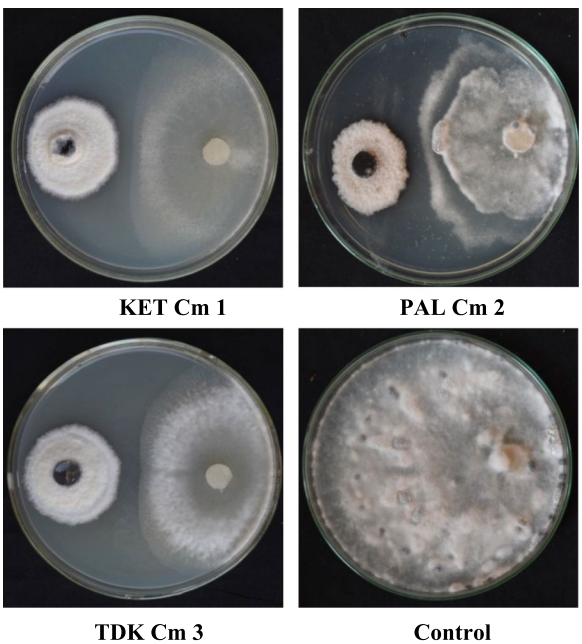


Fig. 10 In vitro screening of *Coniothyrium minitans* against *Sclerotinia sclerotiorum* NAJ SS5 at 20 °C (pre-inoculation)

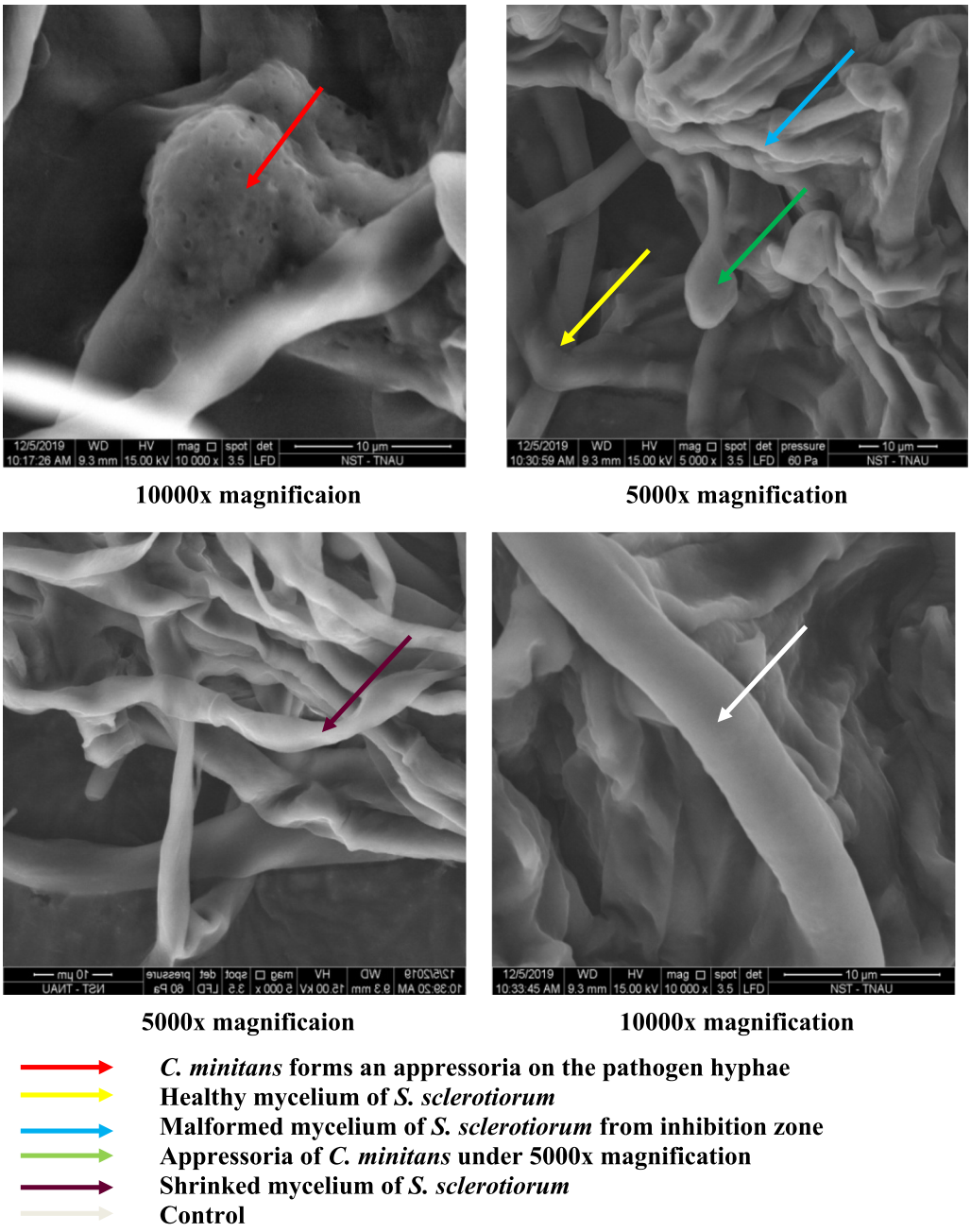


Fig. 11 FESEM images of hyphal interaction between *C. minitans* TDK Cm3 and *S. sclerotiorum* NAJ SS5

Coniothyrium minitans is a promising biocontrol agent of *S. sclerotiorum* both in glasshouse and in field trials (Budge and Whipps 1991). Culturing of *C. minitans* for large-scale production requires standard culture media, with suitable pH and temperature. In the present study, eight different cultural media were tested for their support in the growth of *C. minitans* and its pycnidial production. Among the different cultural media tested, PDA recorded maximum mycelial growth than other cultural media. The result of the present study was supported by the findings of Muthumeenakshi et al. (2001). McQuilken et al. (1995) reported that PDA supported conidial germination, mycelial growth and pycnidial production of *C. minitans*. Papavizas et al. (1984) stated that PDA medium supported hyphal extension of *C. minitans* when compared to molasses yeast

Table 8 In vitro assay for crude metabolites of *Coniothyrium minitans* isolates against *Sclerotinia sclerotiorum* NAJ SS5 using agar well diffusion and paper disc method

S. No.	Treatments	Growth of *Sclerotinia sclerotiorum* (cm)*	Inhibition zone (cm)*	Per cent Inhibition over control **
Agar well diffusion method				
1	KETCm1	1.0	1.5	88.8[a] (70.45)
2	PAL Cm2	1.5	1.0	83.3[b] (65.88)
3	TDK Cm3	1.0	1.5	88.8[a] (70.45)
4	Control	9.0	0.0	0.0
			CD	1.36
Paper disc assay				
1	KETCm1	5.6	2.0	37.7[b] (37.88)
2	PAL Cm2	5.5	3.0	38.8[ab] (38.53)
3	TDK Cm3	5.4	1.0	40.0 [a] (36.54)
4	Control	9.0	0.0	0.0
			CD	1.44

In the column, means followed by a common letter are not significantly different at 5% level by DMRT

*Values are the means of four replications

**Values in the parentheses are arc sign-transformed values

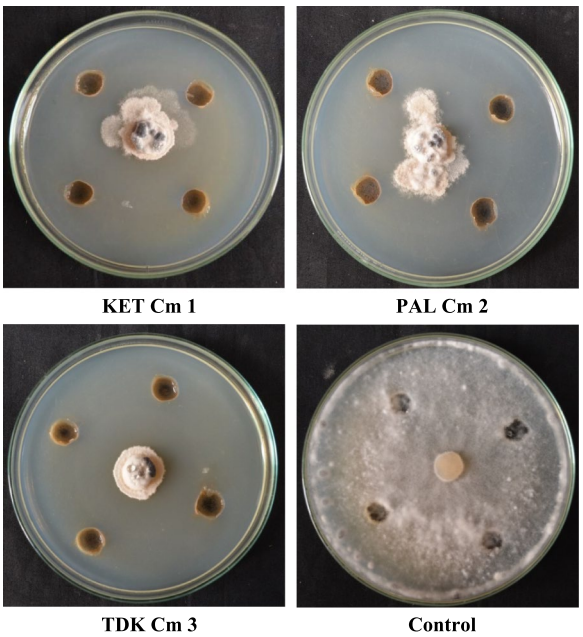


Fig. 12 In vitro assay for crude metabolites of *Coniothyrium minitans* isolates against *Sclerotinia sclerotiorum* NAJ SS5 using agar well diffusion method

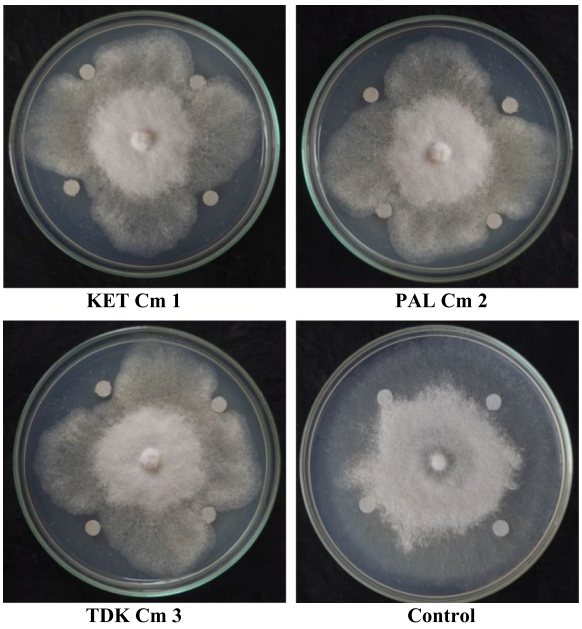


Fig. 13 In vitro assay for crude metabolites of *Coniothyrium minitans* isolates against *Sclerotinia sclerotiorum* NAJ SS5 using paper disc assay

Table 9 In vitro assay for crude metabolites of *Coniothyrium minitans* TDK Cm3 isolate against *Sclerotinia sclerotiorum* NAJ SS5 using poison food technique

S. No.	Concentration (%)	Growth of *S. sclerotiorum* (Cm)*	Per cent inhibition over control**	No. of Sclerotia observed
1	0.25	9.0	0.0[c] (1.62)	47
2	0.50	9.0	0.0[c] (1.62)	13
3	0.75	8.0	11.1[b] (19.46)	0
4	1.0	4.0	55.5[a] (48.16)	0
5	Control	9.0	0.0	45
		CD	0.86	

In the column, means followed by a common letter are not significantly different at 5% level by DMRT

*Values are the means of three replications

**Values in the parentheses are arc sign-transformed values

broth. Similarly, the effect of temperature on the growth of *C. minitans* was studied. Muthumeenakshi et al. (2001) stated that both room temperature (28 ± 2 °C) and 20 °C supported the growth of *C. minitans*. However, high mycelial growth was recorded at 20 °C in various cultural media. A high temperature of 28 ± 2 °C had recorded lesser mycelial growth in different cultural media tested. Mcquilken et al. (1997) studied the

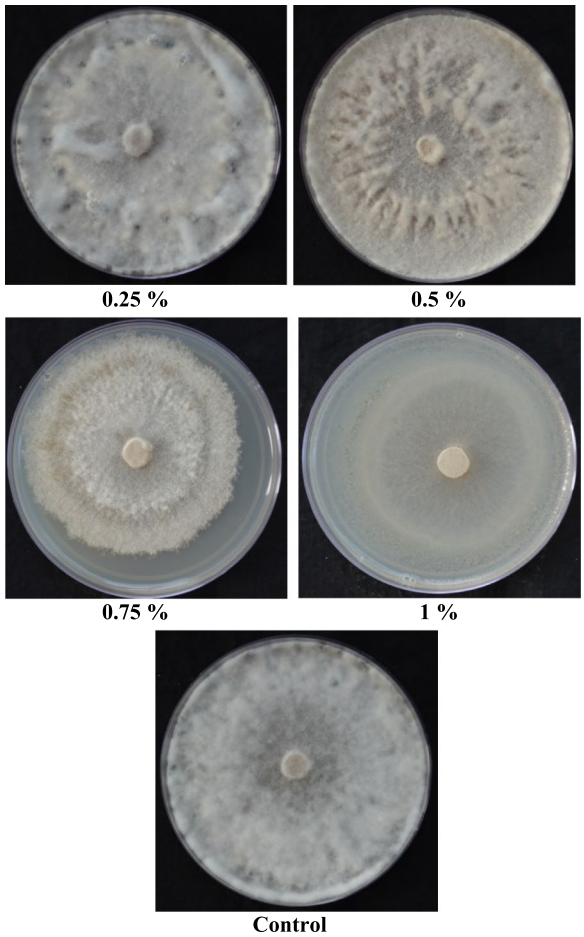


Fig. 14 In vitro assay for crude metabolites of *Coniothyrium minitans* TDK Cm 3 isolate against *Sclerotinia sclerotiorum* NAJ SS5 using poison food technique

temperature range for the mycelial extension of *C. minitans* and found that the temperatures 20 to 25 °C were optimal for mycelial extension in different isolates. They also observed that 20 °C was the optimum temperature for pycnidial production. Similar reports were made by Sandys-Winsch et al. (1993). The result of the present study also is in agreement with the report of the above-said workers. The results on the effect of pH agreed with the report of Mcquilken et al. (1997) who stated that cultural media pH between 4.5 to 6.2 supported conidial germination, hyphal extension and pycnidial production of *C. minitans.* The effect of *C. minitans* isolate on the mycelial growth of *S. sclerotiorum* tested under dual cultural technique as co-inoculation and pre-inoculation. The *C. minitans* isolates reduced the mycelial growth and sclerotial production of *S. sclerotiorum* under two different methods of inoculation. The effectiveness of co-inoculation of *C. minitans* isolates and *S. sclerotiorum* was comparatively less than pre-inoculation method. Approximately 90% increased reduction in mycelial growth was observed in the pre-inoculation method than the co-inoculation. Though the growth rate of *C. minitans* strains was slower than that of *S. sclerotiorum*, the colonies were coalesced and an inhibition zone was observed at the point of contact. Jones et al. (2014) studied the effect of different *C. minitans* on the mycelial growth of *S. sclerotiorum*. In dual culture, using both co-inoculation and pre-inoculation method, they observed that pre-inoculation reduced the mycelial growth comparatively than co-inoculation method. They also observed mycoparasitism of *C. minitans* around the *S. sclerotiorum.* They concluded that *C. minitans* LU112 reduced apothecial production and viability of sclerotia. Similarly, Hoes and Huang (1975) studied the mycoparasitism of *C. minitans* against *S. sclerotiorum* using a dual culture technique. They observed that the lysis of hyphae as well as sclerotia. The parasitized sclerotia decayed and became soft as a result of hyphal disintegration. This may be due to the production of exo- and endoglucanase by the *C. minitans*. Jones et al. (1974) observed the disintegration of *S. sclerotiorum* hypha due to lytic enzymes secreted by *C. minitans*. Jones and Watson (1969) described the production of endo- and exoglucanase by *C. minitans* involved in the breakdown of the cell wall of *S. sclerotiorum*. Boosalis (1956) showed different modes of parasitism by *C. minitans* on the *P. vermiculatum* and *R. solani*. Cell wall lysis and coiling of host hyphae by antagonist *T. harzianum* were observed by Inbar et al. (1994). Similar findings were reported by Vinodkumar et al. (2017) who observed the colonization of *T. asperellum*—NVTA2 on sclerotial bodies of *S. sclerotiorum* through SEM.

Choudary et al. (2007) observed the metabolite produced from *T. harzianum*, which completely inhibited the mycelial growths of *F. oxysporum, S. rolfsii* and *R. solani*, respectively. Similar reports were made by Nagamani et al. (2017) who observed a reduction in the mycelial growth of *F. oxysporum* f. sp. *ciceri* by non-volatile organic metabolites of *Trichoderma* isolates. Vinodkumar et al. (2017) found a good inhibitory effect of cell-free crude extract from *T. asperellum* NVTA2 on the mycelial growth of *S. sclerotiorum*. Maddila et al. (2016) reported the antibacterial and antifungal activity of Benzothiazole compounds.

Derbalah et al. (2012) confirmed the antifungal activity of Tetradecanoic acid. The antifungal activity of phytol acetate was reported by Aziz et al. (2019). The results of the present findings are also in agreement with the report of the above-said workers.

Table 10 Non-volatile organic metabolites (NVOM) associated with the secretome of *Coniothyrium minitans* TDK Cm 3 isolate

S. No.	RT (min)	Peak area (%)	Name of the compound	Mol. formula	Mol. wt (g)	Mol. structure	Function	References
1	3.013	2.443	Decane	$C_{10}H_{22}$	142		Antifungal activity	Bayan and Aksit (2016)
2	6.305	0.298	Benzothiazole	C_7H_5NS	135.19		Antibacterial and Antifungal activity	Flores Sandoval al. (2013)
3	14.973	1.300	Tetradecanoic acid	$C_{14}H_{28}O_2$	228.37		Antifungal activity	Derbalah et al. (2012)
4	15.608	0.854	1-Nonadecene	$C_{19}H_{40}$	268.529		Antimicrobial and Antioxidant activity	Yi et al. (2019)
5	16.444	0.485	Phytol acetate	$C_{22}H_{42}O_2$	338.6		Antifungal activity	Aziz et al. (2019)
6	17.289	0.326	17-Octadecynoic acid	$C_{18}H_{30}O_2$	278.43		Antimicrobial and Antifungal activity	Meena et al. (2017)
7	17.849	2.543	Dimethyl palmitamine	$C_{18}H_{39}N$	269.5		Antimicrobial activity	Chakraborty et al. (2009)
8	18.905	0.637	n-Hexadecenoic acid	$C_{16}H_{32}O_2$	256.43		Antimicrobial and Antifungal activity	Chukeatirote and Jenjai (2018)
9	23.181	0.978	1-Heneicosyl formate	$C_{22}H_{44}O_2$	340.6		Antibacterial and Antiviral activity	Krishnamoorthy et al. (2018)
10	25.382	0.488	1-Heptatriacotanol	$C_{37}H_{76}O$	537		Antifungal activity	Ertürk and Tas (2011)

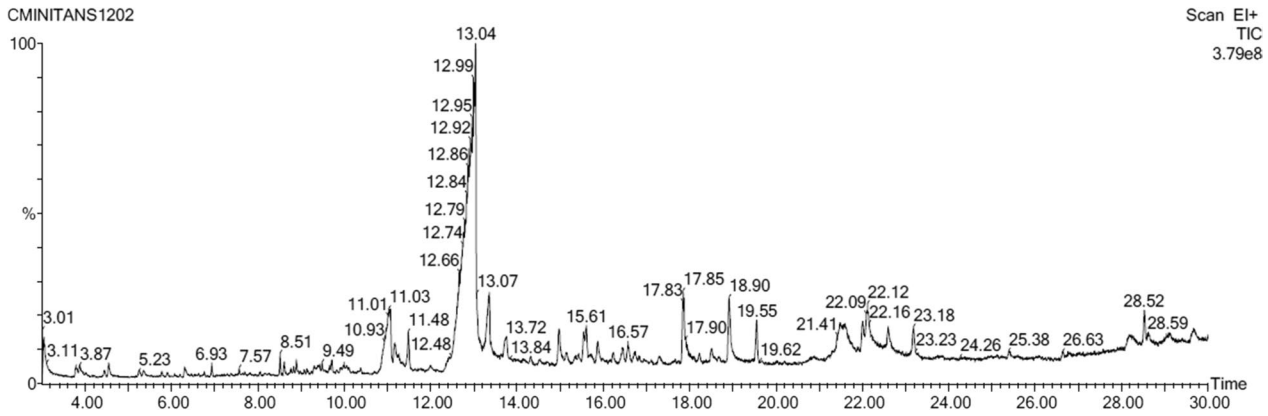


Fig. 15 GC–MS chromatogram of *Coniothyrium minitans* TDK Cm 3 isolate

Conclusion

The present study mainly focused on the *Coniothyrium minitans,* a sclerotial mycoparasite of *Sclerotinia sclerotiorum*, and its morphology, molecular characterization, mode of action and its mechanisms against the pathogen, in vitro evaluation of effective isolates for the management of head rot pathogen. This provided the basic information for further studies to develop the formulations and in vivo evaluations for the management of head rot pathogen.

Abbreviations

KET: Ketti; PAL: Palada; TDK: Thadiyankudisai; PDA: Potato dextrose agar; ITS: Internal transcribed spacer; RNA: Ribonucleic acid; GC–MS: Gas chromatography mass spectrometry; SEM: Scanning electron microscope; ESEM: Environmental scanning electron microscope; PCR: Polymerase chain reaction; PI: Per cent inhibition.

Acknowledgements

The authors gratefully acknowledge the Department of Plant Pathology, Department of Vegetable Science, Tamil Nadu Agricultural University, Coimbatore, for extending the necessary facilities to carry out the experiments.

Author contributions
This work was carried out in collaboration among all authors. KS and MK designed the study. KS performed the laboratory experiments and produced the manuscript. AK and TR revised the manuscript. RS and MK managed the literature searches. All authors read and approved the final manuscript.

Funding
Not applicable.

Availability of data and materials
Not applicable.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
All the authors declared that they have no competing interests.

Author details
[1]Department of Plant Pathology, TNAU, Coimbatore 641003, India. [2]Department of Vegetable Science, HC & RI, TNAU, Coimbatore 641003, India.

Received: 18 June 2022 Accepted: 5 November 2022
Published online: 19 November 2022

References

Adams PB, Ayers WA (1981) *Sporidesmium sclerotivorum*: distribution and function in natural biological control of sclerotial fungi. Phytopathology 71(1):90–93
Aziz SDA, Jafarah NF, Yusof ZNB (2019) Phytol-containing seaweed extracts as control for *Ganoderma boninense*. J Oil Palm Res 31(2):238–247
Bayan Y, Aksit H (2016) Antifungal activity of essential oils and plant extracts from Sideritis germanicopolitana BORNM. growin in Turkey
Boosalis MG (1956) Effect of soil temperature and green-manure amendment of unsterilized soil on parasitism of *Rhizoctonia solani* by *Penicillium vermiculatum* and *Trichoderma* sp. Phytopathology 46:473–478
Budge SP, Whipps JaM (1991) Glasshouse trials of *Coniothyrium minitans* and *Trichoderma* species for the biological control of *Sclerotinia sclerotiorum* in celery and lettuce. Plant Pathol 40(1):59–66
Budge SP, Mcquilken MP, Fenlon JS, Whipps JM (1995) Use of *Coniothyrium minitans* and *Gliocladium virens* for biological control of *Sclerotinia sclerotiorum* in glasshouse lettuce. Biol Control 5(4):513–522
Campbell WA (1947) A new species of *Coniothyrium* parasitic on sclerotia. Mycologia 39(2):190–195
Chakraborty U, Chakraborty BN, Basnet M, Chakraborty AP (2009) Evaluation of Ochrobactrum anthropi TRS-2 and its talc based formulation for enhancement of growth of tea plants and management of brown root rot disease. J Appl Microbiol 107(2):625–634
Choudary KA, Reddy KRN, Reddy MS (2007) Antifungal activity and genetic variability of *Trichoderma harzianum* isolates. J Mycol Pl Pathol 37(2):1–6
Chukeatirote E, Jenjai N (2018) Antimicrobial Activity of Wood Vinegar from Dimocarpus longan. EnvironmentAsia 11(3)
Dennis C, Webster J (1971) Antagonistic properties of species-groups of Trichoderma: I. Production of non-volatile antibiotics. Trans Br Mycol Soc 57(1):25-IN23
Derbalah AS, Dewir YH, El-Sayed AE (2012) Antifungal activity of some plant extracts against sugar beet damping-off caused by *Sclerotium rolfsii*. Annals of Microbiology 62(3):1021–1029
Dhingra OD, Sinclair JB (1985) Basic plant pathology methods. CRC Press, Inc
Ertürk O, Tas B (2011) Antibacterial and antifungal effects of some marine algae. Kafkas Univ Vet Fak Derg 17:S121–S124
Flores Sandoval CA, Cuevas Hernández RI, Correa Basurto J et al (2013) Synthesis and theoretic calculations of benzoxazoles and docking studies of their interactions with triosephosphate isomerase. Med Chem Res 22:2768–2777
Hoes JA, Huang HC (1975) Sclerotinia sclerotiorum: viability and separation of sclerotia from soil. Phytopathology 65(12):1431–1432
Huang HC, Bremer E, Hynes RK, Erickson RS (2000) Foliar application of fungal biocontrol agents for the control of white mold of dry bean caused by *Sclerotinia sclerotiorum*. Biol Control 18(3):270–276
Inbar J, Abramsky M, Cohen D, Chet I (1994) Plant growth enhancement and disease control by *Trichoderma harzianum* in vegetable seedlings grown under commercial conditions. Eur J Plant Pathol 100(5):337–346
Jones D, Watson D (1969) Parasitism and lysis by soil fungi of *Sclerotinia sclerotiorum* (Lib.) de Bary, a phytopathogenic fungus. Nature 224(5216):287–288
Jones EE, Whipps JM (2002) Effect of inoculum rates and sources of *Coniothyrium minitans* on control of *Sclerotinia sclerotiorum* disease in glasshouse lettuce. Eur J Plant Pathol 108(6):527–538
Jones D, Gordon AH, Bacon JSD (1974) Co-operative action by endo-and exo-β-(1→ 3)-glucanases from parasitic fungi in the degradation of cell-wall glucans of *Sclerotinia sclerotiorum* (Lib.) de Bary. Biochemical Journal 140(1):47–55
Jones EE, Rabeendran N, Stewart A (2014) Biocontrol of Sclerotinia sclerotiorum infection of cabbage by Coniothyrium minitans and Trichoderma spp. Biocontrol Sci Technol 24(12):1363–1382.
Krishnamoorthy R, Athinarayanan, J, Periasamy VS, Adisa AR, Al-Shuniaber MA, Gassem MA, Alshatwi AA (2018) Antimicrobial activity of nanoemulsion on drug-resistant bacterial pathogens. Microbial Pathogenesis 120:85–96
Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33(7):1870–1874
Maddila S, Gorle S, Seshadri N, Lavanya P, Jonnalagadda SB (2016) Synthesis, antibacterial and antifungal activity of novel benzothiazole pyrimidine derivatives. Arab J Chem 9(5):681–687
McQuilken MP, Mitchell SJ, Budge SP, Whipps JM, Fenlon JS, Archer SA (1995) Effect of *Coniothyrium minitans* on sclerotial survival and apothecial production of *Sclerotinia sclerotiorum* in field-grown oilseed rape. Plant Pathol 44(5):883–896
Mcquilken MP, Budge SP, Whipps JM (1997) Effects of culture media and environmental factors on conidial germination, pycnidial production and hyphal extension of *Coniothyrium minitans*. Mycol Res 101(1):11–17
Meena M, Swapnil P, Zehra A, Dubey MK, Upadhyay RS (2017) Antagonistic assessment of Trichoderma spp. by producing volatile and non-volatile compounds against different fungal pathogens. Archiv Phytopathol Plant Protect 50(13-14):629–648
Muthumeenakshi S, Goldstein AL, Stewart A, Whipps JM (2001) Molecular studies on intraspecific diversity and phylogenetic position of *Coniothyrium minitans*. Mycol Res 105(9):1065–1074
Nagamani P, Bhagat S, Biswas MK, Viswanath K (2017) Effect of volatile and non volatile compounds of *Trichoderma* spp. against soil borne diseases of chickpea. Int J Curr Microbiol App Sci 6(7):1486–1491
Nene YL, Thapliyal PN (1993) Poison food technique. Fungic Plant Dis Control 2:413–415
Ojaghian MR (2009) First report of *Coniothyrium minitans*, a mycoparasite of *Sclerotinia sclerotiorum*, in Iran. Aust Plant Dis Notes 4(1):75–77
Papavizas GC, Dunn MT, Lewis JA, Ristaino JB (1984) Liquid fermentation technology for experimental production of biocontrol fungi. Phytopathology 74(10):1171–1175
Punithalingam E (1982) Coniothyrium minitans. CMI Descr Pathog Fungi Bact 732:1–2
Sambrook HC (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor
Sandys-Winsch C, Whipps JM, Gerlagh M, Kruse M (1993) World distribution of the sclerotial mycoparasite *Coniothyrium minitans*. Mycol Res 97(10):1175–1178
Sankar P, Jeyarajan R (1996) Biological control of sesamum root rot by seed treatment with *Trichoderma* spp. and *Bacillus subtilis*. Indian J Mycol Plant Pathol 26(2):217–220
Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22(22):4673–4680

Vinodkumar S, Indumathi T, Nakkeeran S (2017) *Trichoderma asperellum* (NVTA2) as a potential antagonist for the management of stem rot in carnation under protected cultivation. Biol Control 113:58–64
Wang A, Lin W, Chen X, Lu G, Zhou J, Wang Z (2008) Isolation and identification of Sclerotinia stem rot causal pathogen in *Arabidopsis thaliana*. J Zhejiang Univ Sci B 9(10):818
Whipps JM (1992) Use of mycoparasites for biological control of *Sclerotinia sclerotiorum* in the Glasshouse. Biological control of plant diseases. Springer, pp 437–441
White TJ, Bruns T, Lee SJWT, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protoc Guide Methods Appl 18(1):315–322
Yi F, Sun J, Bao X, Ma B, Sun M (2019) Influence of molecular distillation on antioxidant and antimicrobial activities of rose essential oils. LWT 102:310–316
Zhang D, Yang Y, Castlebury LA, Cerniglia CE (1996) A method for the large scale isolation of high transformation efficiency fungal genomic DNA. FEMS Microbiol Lett 145(2):261–265

Publisher's Note