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CHARACTERIZATION OF THE FUNGICIDAL ACTIVITY OF *Calothrix elenkinii* USING CHEMICAL METHODS AND MICROSCOPY

© 2012 C. Natarajan*, R. Prasanna*, V. Gupta*, P. Dureja**, and L. Nain*

*Division of Microbiology, Indian Agricultural Research Institute (IARI), New Delhi - 110012, India

**Division of Agricultural Chemicals, Indian Agricultural Research Institute (IARI), New Delhi – 110012, India

e-mail: radhapr@gmail.com

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An investigation was directed towards biochemical characterization of cyanobacterium *Calothrix elenkinii* and analysis of the chemical nature and mode of action of its fungicidal metabolite(s) against oomycete *Pythium debaryanum*. Biochemical characterization of the culture in terms of carbohydrate utilization revealed the facultative nature of *C. elenkinii*. Unique antibiotic markers were also found for this strain. 16S rDNA sequencing of the strain revealed 98% similarity with *Calothrix* sp. PCC7101. The fungicidal activity was tested by disc diffusion assay of different fractions of the culture filtrate. A minimum inhibitory concentration of 10 μl was recorded for ethyl acetate fraction of the 7-weeks old culture filtrates. HPLC, followed by NMR spectral analysis demonstrated the presence of a substituted benzoic acid in the ethyl acetate fraction. Microscopic examination revealed distinct granulation, followed by disintegration of the hyphae of *Pythium* sp., indicating the presence of an active metabolite in the culture filtrates of *Calothrix* sp. The fungicidal activity of *C. elenkinii* can be attributed to the presence of 3-acetyl-2-hydroxy-6-methoxy-4-methyl benzoic acid. This is the first report of a benzoic acid derivative having fungicidal activity in cyanobacteria.

Cyanobacteria comprise the only coherent group of prokaryotes which proliferate both in eutrophic and oligotrophic type of marine and freshwater habitats. Besides these habitats, they are widespread in tropical/temperate rice fields, in which they play a significant role in the maintenance of soil fertility [1]. They have been identified as one of the most promising group of organisms, which produce a wide range of biologically active and novel secondary metabolites such as cyclic peptides, alkaloids and lipopolysaccharides [2–4]. The production of secondary metabolites gives a competitive advantage to these microorganisms for survival in diverse environmental conditions.

Research on biological control of plant pathogens has increased considerably over the past years due to public concern about the use of hazardous chemical pesticides. A number of cyanobacteria belonging to genera *Anabaena*, *Nostoc*, *Microcystis* exhibit algicidal, antibacterial and antifungal properties [3, 5]. The antifungal cyclic peptides – laxaphycin A and B are known to be produced by *Anabaena laxa* [6]. *Calothrix fusca* was found to produce calophycin, a cyclic decapeptide compound of 1248 Da molecular weight, exhibiting fungicidal activity [7].

In this study, we used *Calothrix elenkinii* strain which had shown fungicidal activity against *Rhizoctonia bataticola, Pythium aphanidermatum* and *Pythium debaryanum* [8, 9]. The aim of the study is to investigate the mode of action and chemical nature of the fungicidal metabolite(s) of *C. elenkinii*.

MATERIALS AND METHODS

Strains and growth conditions. *Calothrix elenkinii* strain (CCC 124) was originally isolated from the rice fields of Indian Agricultural Research Institute (New Delhi, India) and tested for fungicidal activity against phytopathogenic fungus *Pythium debaryanum* ITCC 95 taken from Indian Type Culture Collection, Division of Plant Pathology, Indian Agricultural Research Institute. The cyanobacterial strain was axenized by standard procedures employing a set of antibiotics [10]. Cultivation of *C. elenkinii* was done in nitrogen free BG-11 medium [11] at 28 ± 2°C under light: dark cycle (16 : 8 h) and intensity of white light 5W m^{-2}. The fungal culture was grown [8, 9], and maintained in potato dextrose agar at 28°C in BOD incubator (Colto, India)

Characterization of *C. elenkinii*. The antibiotic sensitivity profile of *C. elenkinii* was generated by taking 5 ml of homogenized suspension of the axenic culture, mixing well with 0.8% top agar by vortexing and plated on the solidified bottom agar (1.2%) on Petri dish with diameter (9 mm). The agar plates were allowed to solidify and incubated under optimal conditions of light and temperature. After 5 days, 5 mm discs containing different antibiotics (HiMedia Laboratories, India) were placed on the cyanobacterial lawn and zones were recorded up to 10 days of incubation.

In order to characterize the strain, in terms of carbohydrate utilization pattern, 50 μl of the cell suspension of the axenized late log phase (21 d) culture of *C. elenkinii* was inoculated in each well of HiCarbohy-

drate Kit (HiMedia Laboratories, India). Different sets were incubated in 16 : 8 light: dark cycle and continuous dark conditions (24 h) and colour changes were recorded according to manufacturer's instructions.

The amount of protein in pellet and filtrate of different age culture was determined spectrophotometrically with bovine serum albumin as standard [12].

16S rDNA analysis. DNA isolation was done using Plant Ultra Clean DNA isolation kit (MoBio, USA) and quantified by Alpha Imager Gel Documentation system 1220 (Alpha Innotech Corporation, USA). PCR amplification and agarose gel electrophoresis were performed according to standard procedures [13]. DNA ladders were purchased from MBI Fermentas (Burlington, Canada) and all PCR related chemicals were obtained from Bangalore Genei (India). PCR amplification of the 16S rDNA was performed by the same set of primers as used earlier [14–16]. PCR amplification was done using 10 ng genomic DNA. PCR conditions consisted of initial denaturation at 94°C for 5 min, 30 cycles at 95°C (2 min), 42°C (30 s), and 72°C (2 min), plus one additional cycle with a final 10 min chain elongation. The single bands of desired amplicons were excised from the gels, purified using gel extraction kit (Qiagen, USA) and sequenced directly using the same set of primers as used for amplification. The CAP program was used for sequence assembly [17].

In silico analysis of 16S rDNA sequences of *C. elenkinii* was done by NCBI BLASTN program [18]. Multiple sequence analysis was made with available 16S rDNA sequences of different *Calothrix* species from the NCBI data base, using CLUSTALW software. The phylogenetic tree in the form of dendrogram was generated using the neighbor-joining method and maximum-parsimony algorithms as implemented in the program MEGA package version 4.1 [19].

Disc diffusion assay for antifungal activity. The cell pellet and filtrate of culture of different ages (1 to 7 weeks) were separated by centrifugation at 6.000 g for 10 min. The cells in pellet were broken down by sonication (at output 2 s pulse, 50% duty cycles, output control setting 8 for 2 min using Labsonic L.B. Braun Sonicator Biotech International, Germany) and used to evaluate fungicidal activity by disc diffusion assay [3]. The inhibition zone formed was evaluated as positive for antifungal activity and its diameter (in mm) was measured. The culture filtrates were also tested by the same procedure. The culture filtrates were then partitioned using ethyl acetate as solvent and both the phases were used for disc diffusion assay to determine the nature of solubility of the toxic compound.

Microscopy. Microscopic examination was also undertaken in order to investigate the mode of antifungal action of sample using phase contrast light microscope attached with Canon Power Shot S50 Digital Camera and Canon utilities Remote Capture Version 2.7.2.16 software (Japan). The lactophenol cotton blue stained hyphal specimens were observed at 40X magnification.

Minimum Inhibitory concentration (MIC). MIC was determined in microtitre plates using 50 μl obtained from 1 ml of filtrate generated using 10 mg dry biomass of organism. The samples included culture filtrates and the solvent and aqueous phases of 1 ml culture filtrate extracted with equal volume of ethyl acetate. Different dilutions of the filtrates and extracts were tested keeping the final volume as 50 μl. *Pythium debaryanum* grown in PDA plates was removed and suspended in sterile water (100 mg of fungal hyphae/ml sterile water) before use as inoculum. Microtitre plates were prepared with 200 μl of PDA per well and inoculated with 50 μl of homogenized fungal inoculum. After 1 day of growth at 28°C, 50 μl of different age culture filtrate(s) of *C. elenkinii* were inoculated along with controls of ethyl acetate (50 μl), nystatin (100 U/50 μl) and sterile water (50 μl). Inhibition was observed visually after 2 days of incubation and scored for calculation of MIC and IC_{50}.

Chemical characterization of fungicidal compound. The cell free filtrates (from 1–7 weeks old cultures of *C. elenkinii*) were analyzed using preparative thin layer chromatography (TLC). For partial purification of fungicidal compound, ethyl acetate fraction of cell free filtrate (from 6- and 7-weeks old culture) was condensed using Rotary vacuum film evaporator (Perfit, India) kept at 500 mm Hg at 45°C. The extract was dissolved in acetone and separated by TLC (Silica gel 60, Merck, USA) using different solvent systems containing benzene-acetone (45 : 5) and n-hexane-benzene (1 : 1). The different fractions obtained were eluted using acetone (90%), tested for fungicidal activity against *Pythium* sp. and purified.

Samples were also analyzed by HPLC (Varian Prostar, UK) with an RP_{18} reverse phase column (4.5 cm length) as stationary phase and methanol-water (80 : 20 v/v) as mobile phase maintained at a flow rate of 1 ml min^{-1} with the detector wavelength set at 270 nm.

The spectra were generated using ^{1}H-NMR and ^{13}C-NMR, in the Division of Agricultural Chemicals, IARI, (New Delhi, India). The proton nuclear magnetic resonance spectra were recorded using Varian EM 360L (60 MHz), and Bruker 400 AC (400 MHz) instruments (USA). All 1D and 2D spectra were obtained using the standard Bruker software (Bruker AVANCE-400 NMR with TopSpin 1.3 software). The samples were dissolved in a deuterated $CHCl_3$. Tetra methyl silane $Si(CH_3)_4$ was used as internal standard reference signal. The observed chemical shifts (δ) were recorded in ppm and the coupling constant (J) was recorded in Hz. Several modern NMR techniques were performed in order to elucidate the structure of isolated compound [20].

Biochemical characterization of *C. elenkinii* in terms of utilization of different compounds

Compound	Utilization pattern		Compound	Utilization pattern	
	light	dark		light	dark
Lactose	–	–	Inulin	–	+
Xylose	–	–	Sodium gluconate	–	–
Maltose	–	–	Glycerol	–	–
Fructose	–	–	Salicin	–	–
Dextrose	–	–	Glucosamine	–	+
Galactose	+	+	Dulcitol	–	–
Raffinose	–	–	Inositol	–	–
Trehalose	–	–	Sorbitol	–	–
Melibiose	–	–	Mannitol	–	–
Sucrose	–	+	Adonitol	–	–
L-Arabinose	–	+	α- Methyl-D-glucose	–	–
Mannose	+	+	Ribose	+	–
Rhamnose	–	–	Malonate	+	+
Melezitose	–	–	Sorbose	–	–
α- Methyl-D-mannoside	–	–	Lysine utilization	+	+
Xylitol	–	–	Ornithine	+	+
ONPG	+	+	Urease production	+	+
Esculin hydrolysis	+	+	Phenylalanine deamination	+	+
D-Arabinose	–	–	Nitrate reduction	+	+
Citrate	+	+	H_2S production	–	–

The Liquid Chromatography-Mass Spectroscopy (LC-MS) was carried out using Thermo Electron LC-MS spectrometer (Thermo Electron Corporation, USA). Detection of mass was done by electron spray ionization (ESI) source with Finnigan LCQ tune plus program fitted with MAX-detector. Xcalibur software (Thermo Finnigan LCQ LC/MS/MS Classic, USA) was used for the purpose of identification, quantification and fragmentation of required masses. The MS parameters were optimized in direct infusion mode; spray voltage: 3–5 to 5 kV, sheath gas flow rate 10 ml min^{-1}, auxiliary gas flow rate 5 ml min^{-1}, spray current 0.5, capillary temperature 225°C, capillary voltage 20–35 V and tube lens offset 40–65.

Statistical analysis. Standard deviation (SD) values were calculated using SPSS version 10.0 and depicted in the graphs as error bars.

RESULTS

Biochemical characterization of *C. elenkinii*. The biochemical characterization using chromogenic system revealed that *C. elenkinii* utilized mannose, citrate, lysine, ornithine and ONGP (O-nitrophenyl β-D-galacto-pyranoside) in light and dark conditions of incubation. Besides that esculin hydrolysis, urease production, phenylalanine deamination and nitrate reduction were observed under both the conditions. A very slow rate of utilization of sugars (>48 h) such as galactose, sucrose, L-arabinose, and glucosamine during dark incubation and galactose and ribose during light incubation was also recorded (Table).

The *Calothrix* strain was also characterized in terms of its resistance and susceptibility to a set of 20 antibiotics. The cyanobacterium was found to be highly sensitive to metronidazole (5 μg) and zone of inhibition was observed within 24 h, while carbenicillin (100 μg) led to inhibition of cyanobacterial growth within 3 days of incubation. Distinct zones were produced only after 6 to 9 days of incubation in the case of streptomycin (100 μg), tetracycline (30 μg) and erythromycin (10 μg). On the other hand, *C. elenkinii* was not sensitive to amoxycillin (10 μg), amphothricin (20 μg), ampicilllin (20 μg), chloramphenicol (10 μg), gentamycin (10 μg), kanamycin (30 μg), lincomycin (2 μg), nalidixic acid (30 μg),novobiocin (5 μg), penicillin(10 μg), polymixin B (50, 300 μg), rifampicin (30 μg),spectinomycin (100 μg) and trimethoprim (25 μg).

16S rDNA analysis. The PCR amplification for 16S rDNA of *C. elenkinii* generated a 1197 bp sequence (the sequence has been deposited in GenBank with Accession no. GU29083) which showed 98% sequence similarity with *Calothrix* sp PCC 7101 gene 16S rDNA

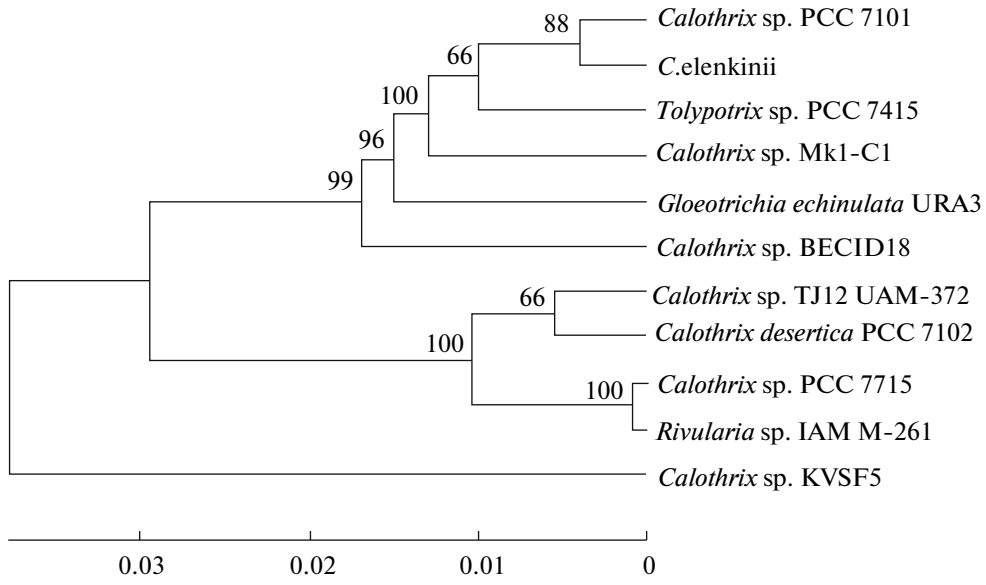

Fig. 1. Phylogenetic tree based on 16S rDNA sequences from different *Calothrix* sp. and related genera using neighbour joining method. The data of sequences other than *Calothrix elenkinii* are taken from GenBank.

partial sequence (Accession No. AB325535.1). Multiple sequence alignment with CLUSTAL W showed that the *C. elenkinii* 16S rDNA sequence obtained in this study was closely related to *Calothrix* sp. PCC 7101 (Fig. 1).

Disc diffusion assay. An investigation was undertaken to test the fungicidal activity of sonicated cell pellet, culture filtrate and ethyl acetate fraction of culture filtrate by disc diffusion assay. The ethyl acetate fraction from 6 and 7 weeks old cultures showed the highest inhibition zone of 16.5 and 17 mm respectively (Fig. 2).

Microscopy. The microscopic examination of the fungal hyphae from the inhibition zone of growth produced by the culture filtrate and ethyl acetate fraction of culture filtrate of *C. elenkinii* was undertaken. Distinct ultra structural changes such as granulation of cytoplasm, degeneration and disintegration of the hyphae and dissolution of internal contents were clearly visible as compared to control (Fig. 3a–3c).

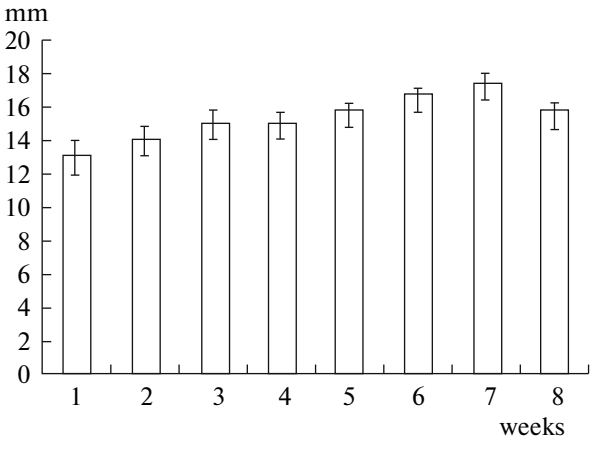

Fig. 2. Influence of culture age on fungicidal activity (measured as zone of inhibition) of *C. elenkinii*.

Minimum Inhibitory concentration (MIC). MIC was observed in 10 μl of ethyl acetate fraction of cell free filtrate of 7-weeks old culture followed by 1.5 μl of cell free filtrate of seven week culture. The IC_{50} was equal to 0.5 μl of ethyl acetate fraction of 7-weeks old cell free filtrate (data not shown).

Chemical characterization of fungicidal compound. TLC analysis of the cell free filtrates from 1–7 weeks old cultures revealed a single band only in the samples from 6- and 7-weeks old cultures. A R_f value of 0.85 was recorded which exhibited fungicidal activity (data not shown). After resolution through HPLC, a compound with retention time of 4.43 min was revealed.

The ^{1}H-NMR spectrum of the HPLC purified fraction revealed the presence of an aromatic proton at δ 6.2 (one proton), δ 3.85 (3H, singlet OCH_3), δ 2.4 (3H, singlet $COCH_3$), δ 1.9 (3H, singlet CH_3). The ^{13}C-NMR revealed peaks of 196 corresponding to carbonyl group (C=O), 176 (COO); 169, 159, 149, 117 and 100 (aromatic carbon); 107 (aromatic CH); 57 aliphatic group (CH_3); 23 (aliphatic CH_3); 12 (aliphatic CH_3). The mass spectrum showed molecular ion peak at m/z 224. Based on these data the corresponding compound responsible for fungicidal activity was identified as 3-acetyl-2-hydroxy-6-methoxy-4-methyl benzoic acid (Fig. 4).

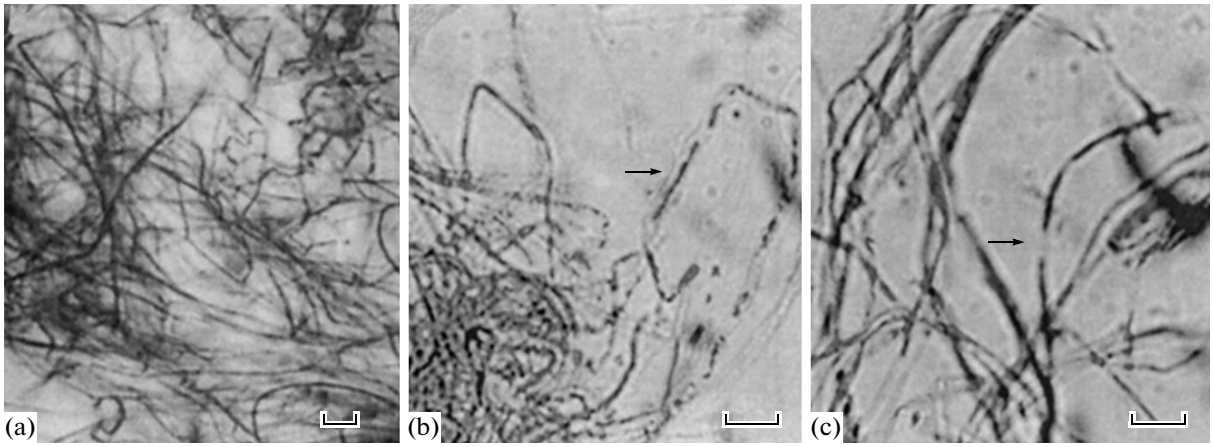

Fig. 3. Microscopic analysis of the effect of cell free filtrate and solvent fraction on the morphology of *P. debaryanum*: (a) control, (b) effect of cell free filtrate, (c) ethyl acetate fraction. The horizontal bars represent 50 μm.

DISCUSSION

Soil borne fungal pathogens are a scourge to most agriculturally important crops and cause severe monetary losses, especially in vegetables. *Pythium* spp. which lead to severe economic losses by causing diseases in seedling nurseries are the most notorious among them. Many cyanobacteria have been found to produce bioactive molecules which show antifungal/fungicidal activity [3]. The genus *Calothrix* is known to be a rich source of valuable bioproducts including pigments and fatty acids, besides biocidal molecules [21]. In the course of our investigations, we have isolated and identified a *C. elenkinii* strain, which exhibits fungicidal activity [9] and its biocontrol potential has been demonstrated against damping of disease of solanaceous vegetables under controlled conditions in the National Phytotron Facility, IARI [8]. Therefore, a need was felt to identify the most potent age of the culture and chemical nature of fungicidal compound, as a prelude to formulating an effective biocontrol agent, using this organism and its metabolites.

Biochemical characterization and antibiotic profiling can serve as markers for *C. elenkinii* for its future use as a biocontrol agent. In our study, the *Calothrix* strain was found to utilize a large number of sugars in light and dark. The genus *Calothrix* is known to be a facultative photoautotroph, with ability to grow in dark under heterotrophic conditions [22], which was in consonance with our studies. The potential to grow heterotrophically in the dark seems to be widespread amongst *Calothrix* sp. than for cyanobacteria in general. The *C. elenkinii* strain was also sensitive to metronidazole, carbenicillin, streptomycin, tetracycline and erythromycin, and resistant to the other antibiotics tested.

A polyphasic approach was utilized to investigate a set of isolates (which included *Rivularia* and *Calothrix* genera) with a high degree of trichome tapering [23]. Analysis of the PC operon (phycocyanin), intervening intergenic spacer (*cpc*BA-IGS) and 16S rRNA gene sequence revealed great heterogeneity and the need for revising the classification of these genera. In our study, 16S rDNA sequencing revealed high level of similarity with the *Calothrix* sp. PCC 7101 sequence available in database.

Disc diffusion assay revealed that 6- and 7-weeks cultures showed the greatest fungicidal activity. This is indicative of the fact that the compound(s) responsible for this activity may be a gradually secreted like chitinase in *Pseudomonas aeruginosa* [24] or a secondary metabolite such as norharmane produced during the late exponential phase or stationary phase in batch cultures of *Nostoc insulare* [25]. Cyanobacterial metabolism depends strongly on environmental factors such as light, temperature, salt concentration and also on the nutrient supply to a greater extent. The production of biocidal compounds by *Anabaena* sp. and *Calothrix* sp. was found to be dependent on the light intensity, temperature, phosphorus concentration and pH of the medium [9]. In older cultures, the change in nutrient concentrations may also lead to qualitative and quantitative changes in the production of secondary metabolites [26]. In the present study, maximal fungicidal activity was observed in ethyl acetate fraction of 7-weeks old culture filtrate (18 mm diameter inhibition zone).

Microscopic observations revealed that the cyanobacterial metabolite from *Calothrix* sp. induced diverse morphological abnormalities in the encountering hy-

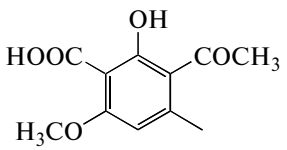

Fig. 4. Molecular structure of the main compound having fungicidal activity isolated from *C. elenkinii* – 3-acetyl-2-hydroxy-6-methoxy-4-methyl-benzoic acid.

phae of the test organism. Such observations have been earlier reported in studies with *Bacillus subtilis* which is known to produce lipopeptides that induce novel structures, composed of aggregates of lipopeptide and solubilized cell wall in fungi [27], and also recorded in *Pythium* sp. [28]. Our microscopic studies of interaction of the metabolite revealed that most fungal hyphae had lost their cytoplasmic content, as reported earlier for *Fusarium oxysporum-Bacillus theobromae* interaction [29].

A major part of the cyanobacterial secondary metabolites are peptides or peptidic substructures, they are synthesized by nonribosomal peptide synthetase or nonribosomal peptide synthetase/polyketide synthase hybrid pathways [30]. Lyngbyabellin B is a cyclic hexapeptide containing two thiazoles and modified octanoic acid [31]. Earlier studies have shown that simple brominated phenols are produced by *Calothrix brevissima* [32]. Other *Calothrix* strains have also been reported to contain heterocyclic quinone compounds which exhibit biocidal activity against malarial parasite and human HeLa cancer cells by inhibiting the RNA polymerase activity [33]. Calophycin, cyclic decapeptide has also been isolated from *Calothrix fusca* which showed antifungal activity against *Aspergillus, Penicillium, Candida* at very low concentrations [7].

This is the first report on the presence of benzoic acid related compounds in cyanobacteria. Benzoic acid is known to exhibit antifungal activity against several fungi, including *Fusarium moniliforme*, *Penicillium* sp. and *Candida* sp. [34, 35]. The presence of benzoic acid derivatives opens up a new perspective on the nature of fungicidal metabolites from cyanobacteria. These substances are widely used in preservation of food as an antifungal agent and in the treatment of fungal skin infections because of their keratolytic activity. Benzoate is also reported to bring about a lowering of intracellular pH, leading to inhibition of phosphofructokinase, glycolysis and growth in yeasts [36]. Therefore, in our investigation, the fungicidal activity can be attributed to the benzoic acid derivative present in the ethyl acetate fraction of the culture filtrate, which may inhibit growth followed by slow disintegration of the fungal mycelia.

Further work is being undertaken to construct a genomic library of *Calothrix elenkenii*, identify, sequence and validate genes responsible for the production of benzoic acid derivative.

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