In Vitro Evaluation of Antagonism of Endophytic *Colletotrichum gloeosporioides* Against Potent Fungal Pathogens of *Camellia sinensis*

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Abstract An endophytic fungus isolated from *Camellia sinensis*, Assam, Northeastern India was identified as *Colletotrichum gloeosporioides* on the basis of morphological characteristics and rDNA ITS analysis. This endophytic fungus was evaluated for growth inhibition against tea pathogens *Pestalotiopsis theae* and *Colletotrichum camelliae*. One isolate of *C. gloeosporioides* showed strong antagonistic activity against *Pestalotiopsis theae* (64 %) and moderate activity against *C. camelliae* (37 %). Fifty percent cell-free culture filtrate from 5-day-old cultures showed highest antagonistic activity against both the pathogens although the inhibition percent was less as compared to dual culture. In the experiment of volatile compounds none of the isolates of *C. gloeosporioides* strains showed visible inhibition against *P. theae* and *C. camelliae*. The activity of extracellular hydrolytic enzymes chitinase and protease was also high in this culture fluid and measured 10 and 4.3 IU/μl, respectively.

Keywords Endophytic fungus · *Camellia sinensis* · Antagonistic activity · Cell free filtrate · Hydrolytic enzymes · rDNA ITS

Introduction

Tea plant (*Camellia sinensis*) is a major cash crop of Assam, North Eastern region of India and tea industry is a backbone of this region's economy. About 14–50 % of crop loss in Assam occurs due to pests and phytopathogens. Among the foliar diseases, grey blight and brown blight caused by *Pestalotiopsis theae* and *Colletotrichum camelliae*, respectively, are economically important. Endophytic fungi live inside the plant without causing any overt negative effect on the host; rather they protect the host plant from pest and diseases [1]. The ability of endophytic fungi of grasses to provide protection from insect herbivores [2, 3] has drawn the attention to study the endophytic microflora especially fungi for better health of crop plants and their bioactivity against wide range of plant and pathogens [4]. Antifungal activity of endophytic fungi against pathogens has been documented well [5–8]. *Colletotrichum gloeosporioides* has been isolated as endophytic fungi from *C. sinensis* in China [9]. *Colletotrichum* sp., *Guignardia* sp., *Pestalotiopsis* sp. and *Aspergillus niger* are considered as dominant species of endophytic fungi in tea garden of Fujian province, China [10]. Apart from tea plant, this endophyte has also been commonly isolated from a wide range of plant species [11, 12].

In tea gardens, various fungicides with different modes of action are being used for the control of foliar diseases. But chemical pesticides not only contribute to their resistance in phytopathogenic fungi, but also cause pollution problems affecting the health of human and animals. Therefore, biological control with endophytes gains importance. However, selection and identification of effective antagonistic organisms is the first and foremost step in biological control [13, 14]. In view of the above mentioned facts, the present study was undertaken to assess

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the antifungal activity of endophytic fungi *Colletotrichum gloeosporioides* isolated from *Camellia sinensis* against potent tea fungal pathogens viz., *Pestalotiopsis theae* and *Colletotrichum camelliae*.

Materials and Methods

Fungal Pathogens

Tea fungal phytopathogen cultures of *Pestalotiopsis theae* and *Colletotrichum camelliae* were obtained from Mycology division of Tocklai Tea Research Station of Assam, India. Both the fungi were grown on PDA (HiMedia, Laboratory Pvt. Ltd., India) plates and incubated at 28 °C for 4–6 days. Stock culture of *P. theae* and *C. camelliae* were maintained on PDA slants.

Isolation of Endophytic Fungi

Healthy and mature leaves of tea plant were cut into small pieces (1 cm²) and the endophytic fungi were isolated as per the procedure described by Petrini and Dreyfuss [15]. The surface sterilized leaves were placed on PDA and the plates were incubated at 28 °C in an inverted position till the hyphal growth of fungi appeared. The effectiveness of surface sterilization was checked according to the method of Petrini [16].

Screening of Endophytic Fungi for Antifungal Activity

The isolated strains were screened for their antagonistic activity against *P. theae* and *C. camelliae* by dual culture method [13]. Mycelial growth (6 mm plugs with the mycelium) removed under aseptic conditions from 4 to 5 day-old pure culture of each isolate of endophytic fungi and pathogenic fungi was transferred to Petri dish (9 cm²) containing 20 ml of PDA and was kept 4 cm apart from each other. The plates were incubated at 28 °C for 8 days and the treatments were replicated in triplicates. The growth of the pathogen and the endophyte was observed constantly and radial growth was recorded by measuring the mean colony diameter on 8th day of inoculation. The percent of inhibition of the test phytopathogenic fungi was calculated using the formula

$$(R1 - R2/R1) \times 100,$$

where R1 was the radial growth of pathogen without endophytic fungi, R2 represent the radial growth of pathogen inoculated with endophytic fungi [17].

Identification of Endophytic Fungi

The fungal cultures were identified based on colony morphology, conidial shape-size and growth rate [18]. Species

level identification of the endophytic fungi was done by ITS rDNA analysis. The fungal genomic DNA was isolated from fresh mycelia grown in PDB (Himedia, India Pvt. Ltd.) using Nucleopore gDNA fungal and bacterial mini kit (Genetix, India) according to manufacturer's protocol. The ITS rDNA was amplified in a thermocycler (Gen Amp 9700, Applied Biosystem, US) using the primer pair ITS1 and TS4 [19]. The PCR conditions were 95 °C for 5 min, 35 cycles of 94 °C for 45 s, 60 °C for 30 s, 72 °C for 45 s and final extension at 72 °C for 5 min. Approximately 575 bp of PCR product was purified using PCR purification Kit (Fermentas, Lithuania) as per manufacturer's instruction. The purified product was sequenced by a Sanger's Dideoxy method on applied biosystem 3730XL (Biolink, New Delhi, India). To identify the isolates, sequences were subjected to BLAST search (<http://www.ncbi.nlm.gov/BLAST>) with the NCBI database [20]. The ITS sequence of the isolate was also aligned with the representative sequence in the NCBI database using CLUSTAL W [21] and employing MEGA 5.2 software, the phylogenetic analysis of the alignment was performed with maximum likelihood method.

Production of Volatile Compounds by Endophytic Isolates

The production of volatile substances by endophytic fungi was determined by growing the culture in sealed Petri plates. Inoculum in the form of 5 mm discs of endophytic fungi and test fungal pathogens were placed in the centre of two separate bottom portions of Petri dish chamber of same size containing PDA, and one of the plates was placed in an inverted position over the other in such a manner they form a chamber. This set up was sealed with parafilm and incubated at 28 °C for 8 days. Control was maintained without endophytic fungi in the bottom plate. Observation was recorded after one week and proportion of inhibition was calculated.

Antagonistic Effects of Culture Filtrate

The isolates of endophytic fungi were grown on PDA plates for 4–5 days at 28 °C in dark. Three pieces of 6 mm mycelial plug of each isolate were cut from the periphery of mycelial growth and inoculated on Erlenmeyer flasks (250 ml) containing 150 ml of PDB. The flasks were then incubated in shaking incubator (Labtech, Korea) at 120 rpm at 28 °C. Mycelial growth was harvested on 5, 8, 11, 14, 17 and 20th day of incubation. Cell-free culture fluid was filtered through Whatman filter paper no. 1 and again re-filtrated through endotoxin-free 0.2 µm PES syringe filter. Radial growth inhibition assay of the test fungal pathogens was performed on PDA plates containing 50 %

(v/v) and 30 % (v/v) cell-free culture filtrate. Fungal growth inhibition was recorded using following formula [22]. A negative control without culture filtrate was maintained.

$$\text{IR}(\%) = \frac{\text{Dc} - \text{Dt}}{\text{Dc}} \times 100$$

where IR is the Inhibitory rate (%), Dc is the Diameter of negative control (cm); Dt is the Diameter of treatment or positive control (cm)

All the experiments were performed in triplicate.

Qualitative and Quantitative Extracellular Enzyme Assay

The isolates of endophytic fungi were tested for their ability to produce the hydrolytic enzymes i.e., cellulase, amylase, protease, lipase and pectinase [23, 24]. Quantitative estimation of proteolytic activity [25] and chitinase [26] was carried out. All assays were conducted in triplicate.

Mycoparasitism

For microscopic observation both pathogen and endophytic fungi were grown in slide culture technique [27]. After 3–8 days of incubation, the region where the hyphae of endophytic fungi and pathogen interacted was observed under light microscope (LEICA DM750).

Test of Significance

Graphpad Prism 5 was used for statistical analysis from which Tukey's multiple comparison test was used for analyzing the data while MEGA 5.2 [28] was used for molecular data analysis.

Results

Isolation and Screening of Endophytic Fungi Against Tea Fungal Pathogens

A total 32 strains were isolated from healthy and mature leaves of tea plants. Among 32 isolates screened using dual culture method, only six strains showed significant antagonistic activity against both the test pathogenic fungi *P. theae* and *C. camelliae*. The degree of growth inhibition ranged from 51.77–63.73 % for *P. theae* and 18.33–36.73 % for *C. camelliae*, respectively (Table 1; Figs. 1, 2). The strain CgloTIN01 showed significantly higher antagonistic activity as determined by Tukey's multiple comparison test ($P < 0.05$) against both the test pathogens as compared to other five endophytic strains.

Table 1 Percent growth inhibition of *P. theae* and *C. camelliae* by endophytic *C. gloeosporioides* isolates after 8 days of incubation

<i>C. gloeosporioides</i> isolates	% of inhibition of <i>P. theae</i>	% of inhibition of <i>C. camelliae</i>
CgloTIN01	63.73 ^a ± 0.43 ^b a ^c	36.73 ^a ± 0.26 ^b a ^c
CgloTIN02	52.77 ^a ± 0.39 ^b c ^c	34.03 ^a ± 0.38 ^b c ^c
CgloTIN03	51.77 ^a ± 0.95 ^b c ^c	30.87 ^a ± 0.93 ^c c ^c
CgloTIN04	53.73 ^a ± 0.43 ^b c ^c	18.33 ^a ± 0.16 ^b e ^c
CgloTIN05	57.77 ^a ± 0.23 ^b b ^c	29.80 ^a ± 0.20 ^b c ^c
CgloTIN06	52.97 ^a ± 0.33 ^b c ^c	25.97 ^a ± 0.06 ^b d ^c

Numbers followed by the ± is standard error (SE)

^a Means the average value of three replicates

^b Standard error of mean

^c Followed by the same letter in a column are not significantly different by Tukey's multiple comparison test ($P < 0.05$)

Fig. 1 Inhibition of *P. theae* by *C. gloeosporioides* in dual culture method after 8 days of incubation

Fig. 2 Inhibition of *C. camelliae* by *C. gloeosporioides* in dual culture method after 8 days of incubation

Identification of Endophytic Fungi

Six fungal isolates were identified as *Colletotrichum gloeosporioides* on the basis of colony morphology, conidia

shape-size and growth rate (Table 2). The analysis of the ITS-rDNA sequence of each six endophytic fungal antagonist showed 99 % similarity with the representative sequence of *C. gloeosporioides* in the NCBI Gen Bank database. The sequence of *C. gloeosporioides* strains designated as CgloTIN01 to NCBI Gen Bank with the accession no KF053197, CgloTIN02 for KF053198, CgloTIN03 for KF053199, CgloTIN04 for KF053200, CgloTIN05 for KF053201 and CgloTIN06 for KF053202. The phylogenetic analysis of 20 aligned representative strains of *Colletotrichum* sp., (NCBI Gen Bank) clustered the strains into two groups; one group having 98 % similarity with the *C. gloeosporioides* (GQ424105 and DQ453993) comparing strains KF053197, KF053198, KF053199, KF053200, and KF053202 and the other second group comparing strain KF053201 showing 100 % similarity with *C. kahawae* (JN715845). The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model [29]. The phylogenetic tree was constructed by Maximum Likelihood method (Fig. 3) and the analysis involved 26 nucleotide sequences.

Effect of Volatile Compounds on the Growth of Pathogens

None of the isolates of *C. gloeosporioides* strains showed visible inhibition due to production of volatile compounds against pathogenic fungi *P. theae* and *C. camelliae* (data not shown).

Table 2 Morphological characteristics of *C. gloeosporioides*

Accession no.	Colony morphology	Conidial shape	Conidial size (μm)	Growth rate (days)
CgloTIN01	White to pale grey mycelium	Cylindrical	14.4–3.6	11.0
CgloTIN02	Black to white mycelium	Cylindrical	13.11–4.3	10.0
CgloTIN03	White mycelium, bright orange conidial masses around the inoculums point.	Cylindrical	12.8–3.87	9.2
CgloTIN04	White to black mycelium	Cylindrical	13.59–3.92	12.5
CgloTIN05	Black to pale grey mycelium, few orange conidial masses near the inoculums point.	Cylindrical	10.05–2.57	13.0
CgloTIN06	Black to white mycelium	Cylindrical	15.45–4.09	12.5

Efficacy of Cell-Free Culture Filtrates of Endophytic Fungi

Culture filtrates of the six endophytic fungal isolates collected after five day of incubation showed maximum inhibition against both the test fungal phytopathogens at 50 % (v/v) concentration (Fig. 4a, b). Significant decrease ($P < 0.05$) in the fungal growth inhibition was recorded at 30 % (v/v) concentration of all cell-free culture filtrate for all the endophytic isolates (Fig. 4a, b). The maximum inhibition of *P. theae* was by the cell-free culture filtrate strain CgloTIN05 followed by CgloTIN06, CgloTIN03, CgloTIN01, CgloTIN02 and CgloTIN04 at 50 % (v/v) concentration. The least antagonistic activity was noted for CgloTIN04 against *P. theae*. Similarly, the maximum inhibition activity against *C. camelliae* was noted with the culture filtrates of CgloTIN04 followed by CgloTIN01, CgloTIN06, CgloTIN02, CgloTIN03 and CgloTIN05, respectively.

Hydrolytic Enzymes by *C. gloeosporioides*

Plate assay carried out for the production of hydrolytic enzymes indicated that all the six endophytic strains of *C. gloeosporioides* produced protease, chitinase and amylase. However production of other enzymes such as cellulase, pectinase and lipase were not detected. The quantitative assay of the 5-day-old cell-free culture filtrates of the strains for hydrolytic enzymes revealed that the strain CgloTIN02 produced highest chitinase activity (Fig. 5). The chitinase activity of CgloTIN01, CgloTIN03 and CgloTIN05 showed significant differences among them. The least chitinase activity was recorded for CgloTIN04. Similarly, protease activity (Fig. 5) of the strains varied from 0.95 to 10 IU/μl. The highest activity was recorded in case of CgloTIN01 and the least protease activity was noted for CgloTIN02.

Interaction of Endophytes with Fungal Pathogens

The isolates of *C. gloeosporioides* showed parasitic behaviour against both the fungal phytopathogens tested by overgrowing and encircling growth habits and spreading spores and ultimately leading to restriction of the growth of the pathogens (Fig. 6).

Discussion

The use of biological control methods to reduce disease incidence caused by phytopathogens is continually being developed and is being used in a variety of crops [30]. Over the last few years, there has been increasing interest in the investigation of endophytic fungi exhibiting substantial and

Fig. 3 The sequence alignment of isolated *C. gloeosporioides* along with different isolates of *colletotrichum* sp. from NCBI was performed using CLUSTAL W program and tree was constructed using the

Maximum Likelihood method in MEGA 5.2 Bootstrap values (1,000) replicates are shown at the nodes. The scale bar represents 0.01 substitutions per nucleotide position

Fig. 4 **a** Percent inhibition of *P. theae* in the presence of 50 and 30 % cell free extract of 5 days of incubation. **b** Percent inhibition of *C. camelliae* in the presence of 50 and 30 % cell free extract of 5 days

of incubation. Followed by same letter between two columns are not significantly different as determined by Tukey's multiple comparison test ($P < 0.005$)

Fig. 5 Chitinase and Protease activity of *C. gloeosporioides* of cell-free culture filtrate of 5 days of incubation

Fig. 6 Microscopic observation of *C. gloeosporioides* and *P. theae* interaction after 8 days of incubation

sustainable antimicrobial activity [31–34]. In the present study, 32 native strains of endophytic fungal strains isolated from tea plants grown in different tea plant gardens of Assam (North Eastern tea growing region of India), were screened against two economically important fungal pathogens, *Pestalotiopsis theae* and *Colletotrichum camelliae* of tea plant. Among the screened isolates strains designated as CgloTINO1, CgloTINO2, CgloTINO3, CgloTINO4, CgloTINO5 and CgloTINO6, showed inhibitory activity against both the test fungal pathogens. However, all the six strains registered their highest antagonistic activity against the fungal pathogen, *P. theae*. The antagonist CgloTINO1 was found to be the most effective against both *P. theae* (63 %) and *C. camelliae* (36 %), as compared to other five isolates (Table 1).

The taxonomic identity of all six strains was ascertained by colony and microscopic morphology [18] and further by ITS rDNA sequencing as *Colletotrichum gloeosporioides*. The ITS rDNA based phylogenetic analysis of six endophytic strains with 26 aligned representative sequences of GenBank of *Colletotrichum* species revealed that the strain designated as CgloTINO5/KF053201 was very much similar to *C. kahawae*/JN715845. This further confirmed the earlier report that, at ITS-rDNA region level both *C. gloeosporioides* and *C. kahawae* have close genetic relationship [35], and, thus can't be differentiated. RFLP analysis of ribosomal and mitochondrial DNA and sequence analysis of *C. gloeosporioides*, *C. kahawae* and *C. fragariae* revealed high degree of molecular similarities between these species and could not be considered as separate species [36–38].

The antifungal activity demonstrated by all the six isolates of endophytic fungi, *C. gloeosporioides* was due to certain diffusible metabolites as revealed by dual culture method. The role of volatile compounds was almost negligible as none of the endophytic *C. gloeosporioides* strains showed growth inhibition of the test pathogens (data not shown). Inhibition of the pathogens by *C. gloeosporioides* was due to overgrowing and restricting the growth of pathogens. Until now there is no report on antagonistic effect for *P. theae* and *C. camelliae* by endophytic *C. gloeosporioides* in dual culture.

Fifty percent cell-free filtrate of endophytic *C. gloeosporioides* showed growth inhibition against both the pathogens and revealed that diffusible metabolites could be responsible for the inhibition. The highest inhibition was shown by the isolate CgloTINO5 (40.33 %) against *P. theae* and CgloTINO4 (37.67 %) against *C. camelliae*. The metabolite, colletotric acid produced by endophytic *C. gloeosporioides* from *Artemisia mongolica* which display antimicrobial activity against bacteria as well as the fungus *Helminthosporium sativum* has been shown [39]. Crude EtOAc extract of endophytic *C. gloeosporioides* showed strong antifungal activity against the phytopathogenic fungi *Cladosporium cladosporioides* and *Cladosporium sphaerospermum* [40]. The compound, Nectriapyrone isolated from endophytic *C. gloeosporioides* and *G. cingulata* possesses strong antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans*, *Trypanosoma cruzi*, *Leishmania tarentolae* and Human *T leukemia cell* [41]. Admittedly, the extracellular metabolites produced by endophytic *C. gloeosporioides* are responsible to inhibit the pathogens.

The chitin degrading enzyme, chitinase and its role in biological control and plant defence mechanism is known [29], [42], [43]. Fungal protease also plays a significant role in cell wall lysis that occurs during pathogen–host interactions [44]. The highest protease activity (10 IU/μl) was

expressed by the isolate CgloTIN01 and chitinase activity (4.3 IU/ μ l) by the isolate CgloTIN02. Culture filtrate from 5-day-old culture was very effective to inhibit the phytopathogens and the activity gradually decreased till 20-day-old culture filtrate (data not shown). The highest growth inhibition of pathogens and maximum enzyme activity in 5-day-old culture filtrate indicated a correlation between the enzyme activity and the inhibition of the pathogens.

The potentiality of *C. gloeosporioides* as biocontrol agent under in vivo conditions from the point of view whether there is any varietal or locational variations in harbouring and its expression against the tested pathogens and on the occurrence of diseases in nature in gardens where the endophyte commonly occurs need to be explored in future.

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