Identification and application of a fungal biocontrol agent *Cladosporium cladosporioides* against *Bemisia tabaci*

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**ABSTRACT**

The entomopathogenic fungus *Cladosporium cladosporioides* is a potential candidate for biocontrol of insect pests. We isolated a strain of *C. cladosporioides* BOU1 from an infected brown plant hoper (BPH) of rice and characterized it using morpho-physiological and molecular analyses. Internal transcribed spacer regions and intervening 5.8S rRNA gene (ITS) sequencing and morpho-pathogenic analyses confirmed that BOU1 is a strain of *C. cladosporioides*. To select the suitable medium for this fungus, a single conidium of BOU1 was grown in potato dextrose agar (PDA), potato dextrose agar with yeast (PDAY), Sabouraud dextrose agar (SDA) and synthetic nutrient-poor agar (SNA) media. The suitable medium for this fungal isolate was determined by fungal growth (colony area and conidiogenesis), and enzymatic activities (protease and lipase). The fungal growth parameters including enzymatic activities showed that the PDA medium is most suitable culture medium for *C. cladosporioides*. Finally, the pathogenicity of this fungal isolate was evaluated against whitefly, *Bemisia tabaci* through direct contact toxicity assay on eggplant leaves by dipping under laboratory conditions. The BOU1 strain caused mortality in *B. tabaci* in a dose-dependent manner, the highest mortality being 71% at 1 x 10^8 conidia/mL. To the best of our knowledge, this is the first report of isolation and molecular characterization of an entomopathogenic fungus *C. cladosporioides* from a BPH of rice. This study suggests that BOU1 is a potential candidate for biological control of whitefly for the promotion of sustainable agriculture.

**Introduction**

*Cladosporium cladosporioides* is a widely distributed cosmopolitan and grey pigmented mold fungus. It was isolated as a saprophyte as well as associated with plants [1–3] and insects [4, 5]. However, the identification of *Cladosporium* spp. only based on morphological features is difficult. Morphologically similar genera of *Cladosporium* spp. have already been distinguished based on morphology [6–8] and DNA phylogeny [9–11] of this species. Several lines of evidence suggest that *C. cladosporioides* is a potent entomopathogenic fungus and thus a potential candidate for biocontrol of insect pests [5, 12].

Colony area and sporulation are important parameters in defining the virulence of entomopathogenic fungi [13]. The extracellular enzymes, viz. protease and lipase, play an important role in the pathogenesis and other physiological processes of *C. cladosporioides* [14, 15]; degrade the major constituents of the insect cuticle that allow hyphal penetration into the cuticle [16, 17]. The entomopathogenic fungus *C. cladosporioides* has been reported to infect different species of insects such as aphid, *Metopolophium dirhodum* (Walker) (Hemiptera: Aphididae) [18]; European pepper moth, *Duponchelia fovealis* (Zeller 1847) (Lepidoptera: Crambidae) [19]; sweet potato whitefly, *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) [4]; two-spotted spider mite, *Tetranychus urticae* Koch (Trombidiformes: Tetranychidae) [5, 12]; carmine spider mite, *Tetranychus cinnabarinus* Boisduval (Trombidiformes: Tetranychidae) [12]; and cotton bollworm, *Helicoverpa armigera* (Hübner 1808) [20].

The present study was conducted for isolation, characterization and molecular identification of the...
entomopathogenic fungus *C. cladosporioides* from an infected brown planthopper of rice in Bangladesh. The influence of different culture media on growth and enzymatic activity of the isolated *C. cladosporioides* BOU1 and its pathogenicity against whitefly (*B. tabaci*) under laboratory conditions were also investigated. This report describes the isolation and identification of a strain of *C. cladosporioides* from an infected BHP of rice and discusses its potential for biocontrol of whitefly.

**Materials and methods**

**Insect, host plant and biocontrol agent**

The sweet potato whitefly, *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) biotype Asia II [21], was obtained from the eggplant, *Solanum melongena*, field of Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU), Salna, Gazipur, Bangladesh. The whitefly population was maintained on eggplant (Family - Solanaceae; variety - Baromashi Singnath Begun; seed source - Sobuj Beej Bhandar, Bangladesh). The biocontrol fungus *Cladosporium cladosporioides* (Davidiellaceae: Capnodiales) (Fresen.) de Vries, strain BOU1 was isolated from a surface sterilized dead brown planthopper *Nilaparvata lugens* Stål (Hemiptera: Delphacidae) from a farmer’s rice field in Gazipur, Bangladesh. The fungus was purified by a single conidium and cultured on potato dextrose agar (PDA) medium.

**Isolation and identification of fungal isolate**

Fungal isolate BOU1 was transferred into a 1.5-mL Eppendorf tube containing sterilized distilled water. Conidial suspension of $1 \times 10^6$ conidia/mL of the fungal isolate was prepared and 100-μL suspension was transferred into the Petri dishes containing 10 mL of PDA. The Petri dishes were incubated at 25°C in a growth chamber. After three days, the dishes were checked daily until the seventh day. The fungal isolate was purified by repeated culturing of the conidia of isolated fungus growing on PDA medium. The water agar medium was also used to visualize the mycelial growth of the isolated fungus (Figure 2E). The morphotaxonomic characteristics of conidia-forming mycelia and conidia structure were identified [22, 23].

For molecular analysis, the genomic DNA of BOU1 was extracted following the modified CTAB (cetyl trimethylammonium bromide) method [24]. The mycelia were harvested from the surface of plate cultures by scrapping and then ground with a mortar and pestle adding 600 μL of extraction buffer (33 mmol/L CTAB; 0.1 mol/L Tris-HCl, pH 8.0; 7.8 mmol/L ethylenediaminetetraacetic acid, EDTA; 0.7 mol/L NaCl). The mycelial suspension was collected in an Eppendorf tube and...
incubated at 65°C for 30 min in a heat block with occasional shaking. The extracted DNA was precipitated with 700 μL of cold isopropanol and washed with 70% ethanol, dried under vacuum and re-suspended in TE buffer (10 mmol/L Tris-HCl, pH 8.0; 1.0 mmol/L EDTA), containing 10 μg/mL of RNase A and incubated at 37°C for 30 min and stored at −20°C. The nuclear rDNA region was amplified by polymerase chain reaction (PCR) [25]. The PCR was performed in a 50 μL reaction mixture with DNA Taq polymerase (Promega, Madison, WI) and purified genomic DNA from the fungal isolate. The PCR amplification reaction was carried out in a thermal cycler (Veriti, Applied Biosystems, ThermoFisher Scientific) as described [26]. The amplification products were subjected to electrophoresis in a 1% agarose gel and stained for 10 min in an ethidium bromide solution (10 μg/mL) and visualized under ultraviolet light (Figure 1). DNA amplification was done by using the primers, ITS1 (5’ TCC GTA GGT GAA CCT TGC GG 3’) and ITS4 (5’ TCC TCC GCT TAT TGA TAT GC 3’) at the concentration of 20 pmol/μL. The amplified regions were sequenced at the National Institute of Biotechnology, Bangladesh. The nucleotide sequence of the isolate was searched for similarity index by using BLAST software. The sequence of ITS1 and ITS2 regions were subsequently submitted to GenBank.

**Influence of different culture media on growth and enzymatic activity of fungal isolate**

The influence of four different culture media, potato dextrose agar (PDA), potato dextrose agar with yeast (PDAY), Sabouraud dextrose agar (SDA) and synthetic nutrient-poor agar (SNA), on the growth and enzymatic activity of the BOU1 isolate of *C. cladosporioides* was investigated. This study was conducted by two approaches: growth (colony area and conidiogenesis) and enzymatic activities (protease and lipase) of the fungus. The measurements of colony diameter and spore production (conidiogenesis) were made daily after inoculation for 14 days. The colony area (cm²) was calculated as follows:

\[
\text{Colony area} = \pi r^2 = \pi \left(\frac{\text{average } d}{2}\right)^2
\]

where, \(r\) is the radius (cm) and \(d\) is the diameter (cm) of the colony.

The activities of two enzymes, protease and lipase, of *C. cladosporioides* were considered as virulence of this
entomopathogen. Protease activity of *C. cladosporioides* was determined as described by Söderhål and Unestam [27] with some modifications. One unit (U) of enzyme activity was defined as the amount of enzyme that, under the assay conditions described, gives rise to an increase of 0.1 units of absorbance in 1 h at 30 °C [28]. Lipase activity of *C. cladosporioides* was determined as described by Pignede et al. [29]. The quantity of fatty acids liberated in samples was determined by equivalents of NaOH used to reach the titration end point, accounting for any contribution from the reagent, using the following equation [30]:

\[
\text{μmol fatty acid/ml subsample} = \left( \frac{\text{ml NaOH for sample} - \text{ml NaOH for blank}}{5 \text{ ml}} \right) \times N \times 1000
\]

where, *N* is the normality of the NaOH titrant used (0.05 in this case). Lipase activity (U/mL) was calculated by determining the amount of supernatant that produces 1 mol of fatty acid per minute under the specified assay conditions.

**Pathogenicity of whitefly by fungal isolate**

The entomopathogenic effects of BOU1 were evaluated against *B. tabaci* through direct contact toxicity assay on eggplant leaves by dipping under laboratory conditions. When plants were 6 weeks old (4-5 true leaves), approximately 50 adult whiteflies (2 days old) were released onto each plant for 48 h to allow egg laying, after which adults were removed. The plants were then incubated for a further 12 days to allow eggs to hatch and reach the stage of second larval instars. Four suspensions of *C. cladosporioides*, 1 × 10⁶, 1 × 10⁷, 1 × 10⁸ and 1 × 10⁹ conidia/mL, were used against the 2nd larval instars of *B. tabaci*. Tween 80, 0.02% without fungal suspension was used for control. Afterwards, infested leaves were labeled and dipped into the suspensions of *C. cladosporioides* on both leaf surfaces for 10 s. There were 10 leaves for each treatment containing 6-10 nymphs. The numbers of living and dead nymphs were recorded (maintained at 8 days 25 ± 1 °C, 70 ± 10% RH and L 12: D 12 photoperiod) after dipping treatments on second instars were performed. Immature ones were considered dead if they had lost their normal yellow-green color, turgidity and smooth cuticle structure.

**Statistical analysis**

The statistical analyses were performed with a one-way analysis of variance (ANOVA) [31] by Proc GLM procedure. Means were separated using Least Significant Difference (LSD) test at 5% level of significance.

**Results and discussion**

Based on colony morphology as well as the size and shape of conidia, the fungal isolate was initially identified as *Cladosporium* sp. (Figure 2). The color of conidia on PDA was grey-olivaceous to dull green or olivaceous-grey, olivaceous-black, and the margins were grey-livaceous to white. Molecular analysis confirmed that BOU1 is a strain of *Cladosporium cladosporioides* (Figure 3). The amplification of the ITS region resulted in a single product (size 507 bp) for the isolate BOU1. The GenBank accession number of the ITS1 sequence of this fungal isolate is MG654669, which was released in December 2017. The ITS1 sequence of BOU1 was very similar to those of accession numbers KY114882, KJ589542 and KJ589553 of the fungus *C. cladosporioides*, an average pair wise similarity of 100% (GenBank).

To find a suitable medium for culturing the fungal isolate BOU1, we tested four media, viz. PDA, PDAY, SDA and SNA, based on growth and two enzymatic activities. Significant differences were observed among the four different media on all variables, viz. colony area (*F* = 66.35, *p* < 0.0001 and df = 3 for the treatments and df = 23 for the total number of observations), conidiogenesis (*F* = 256.54, *p* < 0.0001, df = 3, 23), protease (*F* = 21.51, *p* < 0.0001 and df = 3, 23) and
lipase ($F = 37.49, p < 0.0001$, and $df = 3, 23$) activities of $C. cladosporioides$ (Table 1). The largest colony area, conidiogenesis, protease and lipase activity were $33.5 \text{ cm}^2$, $9.9 \times 10^5$ conidia/mL, $12.8 \mu$g/(mL h) and $9.8 \mu$mol fatty acid/mL, respectively on PDA medium; whereas the smallest colony area, conidiogenesis, protease and lipase activity were $22.6 \text{ cm}^2$, $0.2 \times 10^5$ conidia/mL, $10.9 \mu$g/(mL h) and $4.1 \mu$mol fatty acid/mL, respectively, on SNA medium (Table 1). Although the mycelium of BOU1 covered the surface area in SNA medium, the mycelium was almost invisible by the naked eye in this regard (Figure 4). This indicates that the spore density of BOU1 was very low in this medium as compared with the other three media. This result suggested that SNA media is not suitable for $C. cladosporioides$. All the variables, viz. the colony area, conidiogenesis, protease and lipase activity, were the highest in PDA (Table 1) medium; therefore, we chose PDA as the most suitable medium for in vitro cultivation of BOU1.

The nymph mortality of $B. tabaci$ also showed significant differences among the concentrations of $C. cladosporioides$ ($F = 1154.89, p < 0.0001$ and $df = 3, 23$) as compared with the control (Figure 5). The highest mortality (71%) of $B. tabaci$ was recorded at $1 \times 10^8$ conidia/mL of $C. cladosporioides$ (Figure 5). The mortality percentage of $B. tabaci$ increased with increasing the concentration of $C. cladosporioides$ up to $1 \times 10^8$ conidia/mL; however, the mortality percentage of $B. tabaci$ decreased when the concentration was over $1 \times 10^8$ conidia/mL (Figure 5). This indicates that $1 \times 10^8$ conidia/mL concentration of BOU1 was the most effective concentration for biocontrol of whitefly.

After 10 days of incubation, the culture of $C. cladosporioides$ strain BOU1 produced a white mycelial margin with clumps of more or less verticillate branching conidiophores. These branching conidiophores became colored with the development of the spores. The conidiophores of $C. Cladosporioides$ for a different strain are straight, solitary, unbranched, terminal or lateral and without nodules [3]. These conidia are numerous, in chains of up to nine conidia and also they are limoniform, ovoid, obovoid, aseptate, light brown, hila conspicuous. The colonies of $C. Cladosporioides$ were greenish grey to dark greenish grey, whereas the reverse of the plate revealed a greenish-black coloration [32]. They also reported that the conidia of $C. cladosporioides$ are differing in shape from ellipsoid to thin-walled limoniform, and forming long, branched, pale olive-brown chains, most without a septum, with similar coloring to conidia, and one or no septa. On its own, the morphological key used to identify species of the $C. cladosporioides$ complex [1] did not allow differentiation of the present isolates, thus it was necessary to compare this data with molecular phylogeny [32]. Our results are consistent with the possible presence of cryptic species of $C. cladosporioides$ lineages [1]. A similar phenomenon was also employed for this fungus earlier [3, 32]. The alignments and phylogenetic analysis confirmed the taxonomic identity of BOU1 with $C. cladosporioides$.

The germination rate of $C. cladosporioides$ was highest on PDA medium in our investigation, in contrast to the earlier findings regarding Metarhizium anisopliae [33]. Following the rate of germination, the colony area of BOU1 was also highest on PDA, which was similar to the rate of germination of $M. anisopliae$ [34]. The colony area of Isaria fumosoroseus was highest on high C/N medium [33]. A similar phenomenon regarding the colony area was also reported for Beauveria bassiana and $M. anisopliae$ [35]. Our findings are also similar with the earlier findings [36] that the conidiogenesis of $B. bassiana$ was more effective on PDA medium than on other media, viz. CZA (Czapek’s agar) or SDAY (Sabouraud Dextrose Agar with Yeast Extract). The BOU1 strain has high protease and lipase activities which are linked with its insect killing effects. In this study, the protease and lipase activities of $C. cladosporioides$ were observed as a function of culture conditions and this revealed some interesting results. Both the protease and lipase activities of BOU1 were the highest on PDA medium. Previous findings indicate that the protease (Pr1) activity of $M. anisopliae$ was highest in low and complex C/N ratio media; whereas the lipase activity of $M. anisopliae$ was highest in 2% peptone and osmotic stress media [33].

The hallmark finding of this study is that the native isolate BOU1 significantly killed a major insect pest,
whitefly. The mortality rate of whitefly induced by this isolate was very high as compared to the previously reported cases in different insects, such as 64% mortality in *M. dirhodum* [18]; 48% mortality in *D. fovealis* [19]; and 54% mortality in *H. armigera* [20] when using different isolates of this entomopathogen. The maximum mortality values caused by *C. cladosporioides* in *T. urticae* and *T. cinnabarinus* are reported to be 81.6 and 72.5% after 7 days, respectively [5]. The natural infection of *Cladosporium* spp. on *B. tabaci* nymphs was 87.8% [4]. This entomopathogenic fungus caused mortality of *T. urticae* as high as 70% within 2.77 days [12]. The isolate M16 of *C. cladosporioides* also resulted in failure to hatch for up to 64% of *H. armigera* eggs [20]. Our results suggest that BOU1 is a potential biocontrol agent for whitefly; therefore, it might be useful
for the promotion of sustainable agriculture in Bangladesh.

Conclusions

The fungal strain BOU1 was isolated from BPH of rice in Bangladesh and initially it was characterized morpho-physiologically as Cladosporium sp. Furthermore, by using GenBank, we confirmed that the fungus is C. cladosporioides. Based on the fungal growth parameters, viz. colony area, conidiogenesis, protease and lipase activities, this research revealed that PDA is the most suitable medium for in vitro cultivation of C. cladosporioides BOU1. The BOU1 strain caused mortality in B. tabaci as high as 71% at 1 × 10⁶ conidia/mL. Further study is needed to evaluate the biocontrol efficacy of BOU1 at both greenhouse and field levels before recommending it as a biocontrol agent for the management of whitefly (B. tabaci) in a sustainable way.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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