Mycelial Growth and Biological Control Measures of *Botrytis cinerea* Isolated from Strawberry Fruit Rot Disease in Bangladesh

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**Abstract** Fruit rot disease of strawberry caused by fungus is most economically important disease in Bangladesh, which reduces the production and quality of strawberry at both pre and post-harvest period. Present study was conducted to identify the causal organism of the fruit rot disease of strawberry through classical and molecular techniques and its eco-friendly control measures. A fungal pathogen causing fruit rot was isolated and identified as *Botrytis cinerea* through classical fungal taxonomy and molecular characterization based on their internal transcribed spacer (ITS) region of ribosomal DNA (rDNA). Sequence analysis showed that 5.8S of rDNA sequences were identical. The reciprocal homologies of the ITS region sequences ranged from 98 to 100%. Experimental results suggested that studied fungus (MH371474.1) was genetically similar with MF521935.1 *Botrytis cinerea*. Isolated fungus was evaluated on six different culture media and at five different temperature conditions. The optimum mycelial growth of *B. cinerea* was found on Richard agar medium at 25°C temperature at 7days post inoculation (dpi), while mycelial growth was drastically reduced the temperature 30°C and above. Three *Trichoderma* species viz., *T. ressei*, *T. harzianum* and *T. asperellum* were used to assess the antagonistic effect on isolated pathogenic fungus in which the mycelial growth of it was inhibited mostly by *T. ressei*. Experimental results revealed that *Trichoderma ressei* was the effective antagonistic fungus against *Botrytis cinerea* for the biological control.

**Keywords** *Botrytis cinerea*, Fungal Biology, Molecular Identification, Strawberry

1. Introduction

Strawberry is a stoloniferous perennial herb its cultivation and environmental conditions are very suitable in Bangladesh. Presently strawberry fruit is very popular in our country due to its nutritional values, test and attractive colour. Flowering in strawberry plant is greatly influenced by day length and temperature. For better strawberry production photoperiod 10-20h days temperature 12-30°C and number of short days 12 -24 are essential [1]. Bangladesh day temperature is 15 -25°C, photoperiod 12-16h and short days about 30 -50 days [2]. Bangladesh Agricultural Research Institute (BARI) and Bangladesh Agricultural Development Corporation (BADC) has released strawberry variety namely, BARI strawberry-1 and BADC strawberry. Therefore, Bangladeshi strawberry varieties are grown in different regions of the country during winter season [3].

Fungal disease of strawberry mainly attacks on leaves, fruits, crown and roots. *Colletotrichum gloeosporioides* causing anthracnose leaf spot [4], *Phomopsis obscurans*
causing phomopsis leaf blight [5]. A large number of strawberries have been destroyed every year due to strawberry pre and post-harvest diseases. Anthracnose disease of strawberry caused by Colletotrichum gloeosporioides has been described in Bangladesh [6].

Fruit diseases are caused by Botrytis cinerea causing fruit rot [7], Colletotrichum gloeosporioides causing anthracnose fruit rot [4]. Leaf blight and fruit rot disease of strawberry caused by Curvularia spp. had been discovered in Kurdistan province, Iran, in 2013. Symptoms have been found as small, brown to black, sunken lesions on fruits and brown to black spots with irregular borders on the leaves. Forty isolates have been recovered from infected plants, among them thirty one isolates have been identified as belonging to the genus Curvularia [8].

Molecular identification of fungi through DNA barcoding has become important part of mycological research. It has recently been accepted as the official primary barcoding marker for fungi. Primers like ITS1-F, ITS and ITS5 is biased towards the amplification of basidiomycetes and ITS2, ITS4, ITS5 for the ascomycetes [9]. Recent molecular phylogenetic studies have demonstrated that the ITS region of genomic DNA is very useful for assessing phylogenetic relationships at lower taxonomic levels [10]. Considering the above facts, the present research has been undertaken to isolate and identify of strawberry pathogen using morphological and molecular techniques, to investigate the fungal biology and to evaluate the antagonistic fungal effect against Botrytis cinerea.

2. Materials and Methods

2.1. Sample Collection

Diseased strawberry fruits with characteristic symptoms were collected in to labeled zip lock bag from the field of Botanical Garden at Jahangirmagar University. Experiments were conducted in the Laboratory of Mycology and Plant Pathology, Department of Botany, Jahangirmagar University, Savar, Dhaka, Bangladesh.

2.2. Fungus Isolation and Morphological Characterization

Fungus was isolated through tissue planting method. Infected parts of strawberry fruits were cut in to small pieces about 0.5cm in length in such a manner so as to include both fungal infected and non-infected tissues in piece. Then sterilization was done using NaOCl (5%) solution for 5 minutes and rinsed with distilled water several times. Four pieces of samples were placed into potato dextrose agar (PDA) medium and were incubated under 12/12 hours dark and light condition at 25±2°C for 10 days. Mycelial growth of growing fungus colony was transferred to fresh PDA plates as well as PDA slants to obtain a pure culture. The pure culture of the isolated fungus was identified microscopically using standard methods based on colony morphology, mycelium and conidia [11].

2.3. Molecular Characterization

For molecular characterization, the fungal DNA was isolated by using Promega Wizard DNA Extraction Kit (USA). The ITS region of the rDNA of the fungus was amplified by PCR using universal primers ITS4 (5′-TCTTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGGAAGTAAAAAGTCTGTAACAAGG-3′) according to White et al. [12]. GoTaq G2 Hot Start Green Master Mix which contains GoTaq G2 Hot Start Polymerase, dNTPs, MgCl2 and reaction buffers; was used for PCR reaction. The PCR reaction was performed with 20ng of genomic DNA as the template in a 25µl reaction mixture by using GoTaq G2 Hot Start Green Master Mix as follows-activation of Taq polymerase at 94°C for 5 minute, 35cycles of 94°C for 30Sec, 57°C for 30Sec, and 72°C for 5 minutes each were performed, finishing with a 10-minute step at 72°C. The Maxwell® 16 DNA Purification Kits were used to purify the amplification products (Promega, USA). The purified PCR products of 541bp were sequenced by using 2primers in First BASE Laboratories SdnBhd (Malaysia). Sequencing data were assembled and compared with similar DNA sequences obtained from NCBI GeneBank data base. The phylogenetic analysis was conducted using the multiple sequence alignment tools using MEGA6 software. Maximum likely hood and Neighbor-joining tree were generated using MEGA6 software.

2.4. Effect of Culture Media and Temperature

Six different culture media viz., potato dextrose agar (PDA), carrot agar (CA), potato sucrose agar (PSA), Richard agar (RA), Honey peptone agar (HPA), Honey agar (HA) were used to assay the mycelia growth of the fungus. Different temperatures (15, 20, 25, 30 and 35°C) were maintained for the mycelia growth of the fungus on PDA in an incubator. The mycelia growth was recorded at 7 days post inoculation (dpi). The effect of culture media and temperature on the mycelia growth measurements was described by Sanjuti et al. [13].

2.5. In vitro Mycelial Growth Inhibition of the Pathogen

Mycelial growth inhibition of the fungus was determined using dual culture technique in which biological control agents namely- Trichoderma asperellum voucher JUF0025 (MH368120.1), T. reesei voucher JUF0026 (MH368149.1) and T. harzianum voucher JUF0028 (MH368147.1) were used. An agar disc (6-mm) of the antagonist, Trichoderma spp., was placed 2cm away from the periphery of the Petri dish, and a same sized agar disc of the test fungus, Botrytis cinerea was similarly placed 2cm away from the edge of the Petri plate but on the
end opposite of _Trichoderma_ sample. All pairings were carried out in quadruplicate and incubated at 25°C. Antagonistic activity was tested 7 days after incubation by measuring the radius of the _B. cinerea_ colony in the direction of the antagonist colony. The percent growth inhibition of the fungus was calculated following standard formula [14].

### 2.6. Statistical Analysis

Data generated during the experiment were checked for normality and homogeneity of variance. Data on effects of media, temperature and antagonistic fungi on mycelia growth of the studied fungi was found to be normal and analyzed using one-way ANOVA with Duncan’s Post-Heoctestin SPSS-20.

### 3. Results and Discussion

The mycelium of _Botrytis cinerea_ was composed of brownish olive hyphae. Hyphae were septet. It was characterized by abundant hyaline conidia (asexual spores) borne on grey, branching tree-like conidiophores. The conidia were ellipsoidal or ovoid in shape. They were dry and hydrophobic, colorless, smooth. The fungus also produced highly resistant sclerotia as survival structures in old cultures. They produced olive coloured colony (Figure1). They caused Botrytis fruit rot.

![Figure 1. A: Symptoms of fruit rot disease caused by _Botrytis cinerea_ in the field condition; B: Vegetative growth on PDA medium; C: Microscopic view of _Botrytis cinerea_.](image)

The ITS region was amplified using ITS4 and ITS5 primers and sequenced. Phylogenetic tree based on the nucleotide sequences of the ITS regions in twenty nine fungal taxa were selected from the NCBI data base for phylogenetic analysis. In maximum parsimony tree there are five different clusters were found in the phylogenetic tree. Percent homology of rDNA sequence of ITS region (MH371474.1) was compared with formerly identified fungi KU992700.1 _Botrytis cinerea_, KT271762.1 _Botrytis cinerea_, KU992696.1 _Botrytis cinerea_, KU992697.1 _Botrytis cinerea_, KU992698.1 _Botrytis cinerea_, KU992699.1 _Botrytis cinerea_ and MF521935.1 _Botrytis cinerea_ (Figure 2). Reciprocal homologies of the ITS region sequences ranged from 98 to 100%. The sequencing data of the selected NCBI GeneBank strain (AJ716297.1 _Botrytis hyacinthi_ and AJ716297.1 _Botrytis hyacinthi_) were used as control strain for the comparative studies on phylogenetic relationships with the selected strain of _Botrytis cinerea_ (MH371474.1). The results indicated that all the individual species of _Botrytis cinerea_ belongs to single major cluster. The ITS region is relatively short and can be easily amplified by PCR using universal single primer pairs. Genetic distance exhibited high level of similarity with identical ITS sequences. The size variation was caused by differences in the number of nucleotides, revealing that these strains are clearly distinguishable from each other based on the ecological distribution, substitution, and insertion or deletion polymorphisms of the base position [15]. Alam _et al._ [16] reported that ITS sequences are genetically constant or show little variation within species, but vary between species in a genus. The genetic diversity detected within groups is probably due to an efficient gene flow and to a high genetic compatibility within the strains tested. These results are supported and comparable to the study made by Sikder _et al._ [17].
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The effect of six different solid media viz., PDA, PSA, CA, RA, HPA and HA for the mycelia growth of *Botrytis cinerea* was tested under in vitro condition and the result have been represented in Figure 3. There was statically difference among the culture media to support the growth of the fungus was observed. The maximum mycelia growth (49.83 mm) was found on RA media, followed by HPA media (45.33 mm), and most extensively used PDA medium gave 37.33 mm growth; the lowest vegetative growth (12.17 mm) was found on CA medium. Earlier report showed that only a few culture media types, which supported the radial growth, dry weight growth and sporulation of the fungus, have been mentioned to favor the isolation of *Botrytis fabae*, the nutrient requirements for good growth of the fungus do not imply the nutrient requirements for good sporulation [18]. The overall results of this study indicated that cultural characteristics, mycelial growth and sporulation of *Botrytis fabae* were influenced by media sources, implying that chickpea dextrose agar (CDA) and soybean dextrose agar (SBDA) were best suited for mycelial growth.

**Figure 2.** Maximum parsimony tree of the ITS region of rDNA sequence of the studied organism using neighbor joining method with 1,000 boot strapping. Our organism (MH371474.1) marked with This study.

**Figure 3.** Effect of culture media on vegetative growth of *Botrytis cinerea* at 7 dpi. Data represents as mean ± Standard error (SE) of nine replications. PDA: Potato dextrose agar, CA: Carrot agar, RA: Richard’s agar, PSA: Potato sucrose agar, HPA: Honey peptone agar and HA: Hansen’s agar.
In the current study, *B. cinerea* was found very sensitive to temperature conditions. There was full growth (90 mm) of the Petri plates by the fungus at 25°C temperature, but below ten per cent growth was observed at 35°C (Figure 4). Our results completely supported by earlier workers. Terefe *et al.* [19] reported that *Botrytis fabae* grew faster at 22°C and relatively slower at 26°C. The optimum temperature for the development of gray mold rot of cucumber leaves was between 15°C and 20°C. The disease did not develop over 25°C. Moreover, Ciliberti *et al.* [20] investigated on effects of environment and *Botrytis cinerea* strain, and their interaction on the infection of mature grape berries. The combined effect of temperature (5 to 30°C) and wetness duration (WD) of 3, 6, 12, 24, and 36 h were studied by inoculating berries with conidia. At WD of 36 h, disease incidence was approximately 75% of affected berries at 20 or 25°C, 50% at 15°C, and 30 to 20% at 30 and 10°C; no infection occurred at 5°C.

The antagonistic effect of fungal isolate of *Trichoderma reesei*, *T. harzianum* and *T. asperellum* were assessed on vegetative growth of *B. cinerea* at 7dpi at room temperature using dual culture technique. The results have been represented in Figure 5. The highest mycelial growth inhibition (44.26%) of *Botrytis cinerea* was observed due to *T. reesei* which is similar to *T. asperellum* and the lowest growth inhibition (21.29%) was measured due to *T. harzianum*. In our results, the per cent inhibition growth of *B. cinerea* was found by bio-control agents to certain extent which are in compliance with results of Magdy *et al.* [21] who studied on the antagonistic efficiency and myco-parasitic activity of *T. reesei* against *Botrytis* species, viz., *B. cinerea*, *B. allii* and *B. fabae*. Significant effect was found against *B. cinerea* and *B. fabae*. Singh *et al.* [22] investigated seed treatment with bio-control agents for the control of seed borne infection of chickpea caused by *Botrytis cinerea*, maximum germination (80%) was observed with *T. viride* followed by *T. harzianum* (76.6%) and *P. fluorescens* (70%). Lowest disease incidence was observed in seed treatment with *T. viride* and *P. fluorescens*, followed by Amistar. In another study, cell suspension cultures of grapevine treated with an elicitor from *T. viride* produced increased amounts of resveratrol, a phytoalexin with growth inhibitory activity towards *B. cinerea* [23]. Root colonization by *T. harzianum* T 78 significantly reduced *B. cinerea* infection in tomato wild-types, but not in mutants impaired in the biosynthesis of Jasmionic acid or in the accumulation of salicylic acid or absic acid, thus indicating a role for these plant hormones in the ISR response [24]. On the basis of the experimental results on molecular characterization it could be concluded that tested strains identified as *Botrytis cinerea*, which causes fruit rot disease of strawberry.

**Figure 4.** Effect of temperature on mycelial growth of *Botrytis cinerea* at 7 dpi. Data represents as mean ± Standard error (SE) of nine replications.

**Figure 5.** Antagonistic effect of fungal antagonists on the mycelial growth inhibition (%) of *Botrytis cinerea* at 7 dpi. Data represents as percentage value of nine replications.

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