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Research Article

Collection, isolation and characterization of *Sclerotinia sclerotiorum*, an emerging fungal pathogen causing white mold disease

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Abstract

Sclerotinia sclerotiorum (Lib.) de Bary caused white mold disease with a wide distribution worldwide. For the control of the disease, it is fundamental to understand the identification, morphology, and genetic diversity of the fungus. The objective of this study was to collect and characterize *S. sclerotiorum* isolates from different regions of the country. The characteristics evaluated for the mycelium characterization were: the time required for the fungus to occupy the plate; density of the formed mycelium; coloration of the colonies and mycelia growth rate. Sclerotia assessments were based on the time for the formation of the first sclerotia total number formed per plate, the format of distribution in the plate, and the shape of the sclerotia formed by the isolates. Variability was observed for colony colour, type of growth, the diameter of mycelia growth, sclerotia initiation, and number and pattern of sclerotia formation among the isolates. The evaluated populations presented wide variability for the cultural and morphological characteristics, being predominant in the whitish colonies with fast-growing habitats. The majority of isolates produced a higher number of sclerotia near the margin of the plates and with diverse formats. Phylogenetic analysis revealed that the isolates belonged to a similar group of publicly available *S. sclerotiorum* and were dissimilar from the group of *S. minor*, and *S. trifolium* and distinctly differ from *S. nivalis* group. The present study is the first evidence for morphological and genetic diversity study of *S. sclerotiorum* in Bangladesh. Therefore, this report contributes to more information about the morphological and genetic diversity of *S. sclerotiorum* and can be useful in implementing effective management strategies for the pathogen which caused white mold disease.

More Information

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Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is commonly recognized as a facultative parasitic fungus, causing white mold disease in many crops. The fungus is one of the most important and devastating soil-inhabiting necrotrophic and non-host specific nearly cosmopolitan in its distribution with a broad host range [1]. The fungus infects more than 500 cultivated and wild plant species of angiosperms and gymnosperms [2,3] and causes substantial damage to its host under favorable environments. The pathogen produces sclerotia, which survive for long periods and attack the roots of growing and mature plants, resulting in root rot, basal stem canker, and wilt [4]. *Sclerotinia* Stem Rot (also known as white mold or Sclerotinia Stem and Root Rot) is one of the most important soil-borne diseases. Plant infection occurs either by myceliogenic germination of sclerotia or by ascospores released from apothecia during carpogenic germination of sclerotia. The myceliogenically germinating sclerotia are the main source of infection in processing crops leading to the rotting of aerial parts of the plant in contact with soil [5,6]. The disease can cause disastrous crop failure as disease incidences have been recorded from 60% - 80% and variable yield losses ranged from traces to 100% in several economically important crops worldwide [1,7]. Low temperatures, between 18-23 °C, and high humidity conditions, favor the occurrence of the pathogen. However, the use of contaminated and/or infected seeds, continuous crops in monoculture, a succession of crops with susceptible varieties, mild nocturnal temperatures (below 18 ºC), prolonged rains during cultivation, excessive nitrogen fertilization, and uncontrolled irrigation water supplied [8-10] cause white mold to spread, assuming

great economic and social importance. Due to the abundant production of sclerotia, which allows for the survival of the fungus in the soil for more than 10 years, white mold is considered a disease difficult to control [11]. For sustainable management of diseases, it is essential to understand the etiology, epidemiological conditions, and aggressiveness of the pathogen isolates [12]. There is still relatively little information on the etiology, morphology, and genetic diversity of *S. sclerotiorum* in the literature, especially in Bangladesh. Morphological characteristics of *S. sclerotiorum* isolates collected from various hosts have already been reported in several studies in the country [13,14]. Dickson, [15] studied 33 isolates and found a difference between the density of mycelium and mycelia growth rates. Morral, et al. [16] studied 114 isolates of *S. sclerotiorum* collected from 23 hosts in Canada and found variations in colony color, mycelia growth rate, size, and shape of sclerotia. Corradini, [17] observed large variability in the growth and diameter of colonies, type, and color of mycelium, and production, weight, and distribution of sclerotia in 19 isolates from the Alto Paranaíba-MG region. Pariuad, et al. [18] evaluated the aggressiveness of the *S. sclerotiorum* isolates of different forms viz. by the infection efficiency, by the latent period, the spore production rate, and by the size of the lesion. Lehner, et al. [19] compared the aggressiveness of 20 *S. sclerotiorum* isolates and determined the relationship between aggressiveness and variability. Several molecular methods such as amplified fragment length polymorphism [20], random amplified fragment length polymorphism [21,22], microsatellite marker [23], sequence-related amplified polymorphism (SRAP) technique [24] and Universal Rice Primer Polymerase Chain Reaction (URP-PCR) [25] were used to determine the genetic diversity of fungus. Hence, there is a need to find out the diversity analysis of *S. sclerotiorum* infecting different host plants in Bangladesh for the development of sustainable management technologies. Therefore, the present study was conducted to ascertain the cultural, morphological, and molecular variability among different isolates of *S. sclerotiorum* obtained from infected several hosts in different regions of Bangladesh.

Materials and methods

Collection, isolation, purification, and multiplication of *S. sclerotiorum* isolates

A minimum scale survey for white mold disease was conducted in the country during 2016-17 and 2017-18 cropping years. A total of one hundred and eighty isolates of *S. sclerotiorum* were excised from infected samples collected from 12 different districts. Symptoms of white mold disease on different crops were studied. Diseased plant samples and sclerotia were collected from different host plants in different regions of the country and stored under laboratory conditions in the Plant Pathology Division, Bangladesh Agricultural Research Institute, Gazipur. The infected plant parts with some healthy portions were cut into small pieces followed by surface sterilized with sodium hypochlorite solution (0.2%) and was rinsed with sterilized distilled water 2-3 times. Then, the cut pieces were transferred in 9 cm Petri dishes containing 10-15 ml acid potato-dextrose agar (APDA). The Petri dishes will be incubated for 3-4 days in the dark at 25 ± 1 °C. On the other hand, the sclerotia were placed on a potato dextrose agar (PDA) medium after surface sterilized with sodium hypochlorite solution (0.2%) and incubated at 25 ± 1 °C for 3 days [26]. Each isolate was purified by transferring the single hyphal tip onto the fresh medium and preparing the pure culture of each isolate which was further multiplied.

Cultural and morphological variability

Mycelia disc of 5 mm diameter of each isolate was taken from actively growing colony of 4 days old culture and was transferred on to fresh PDA in Petri plate (90 mm diameter). All the cultures were incubated at 22 ± 1 °C in the incubator and observations of the cultural characters viz., the mycelial linear growth (mm) was recorded at 48 and 72 h while colony color and type of growth were recorded after 72 h after incubation. Four replications with three Petri plates per replication were used for each isolate. The morphological methods as suggested by Morrall, et al. [16] were used for the sclerotia formation i.e., initiation of sclerotia formation in days after incubation (DAI), number of sclerotia formation in plates, and pattern of sclerotia formation on PDA in Petri plates.

Molecular variability of *S. sclerotiorum*

The mycelium of each isolate was grown in potato dextrose broth by incubating at 22 ± 1 °C and 120 rpm. After 5–6 days, the mycelium of each isolate was filtered through Whatman filter no. 1, washed twice with the TE buffer, blot dried completely, and stored at − 70 °C till DNA isolation.

Total genomic DNA was extracted as described by Toda, et al. [27] from each isolate separately using the Wizard Genomic DNA extraction/ purification kit. The quantity and quality of DNA samples were tested by submerged horizontal agarose gel (0.8%) electrophoresis [28] along with a standard marker. Polymerase chain reaction (PCR) was conducted with forwarding primer ITS4 (TCCTCCGCTTAT TGATATGC) and reverse primer ITS5 (GGAAGTAAAAGTCGTAACAAGG) [29] to amplify rDNA-ITS regions of the fungal isolate using commercial Master mix kit (Promega) following manufacturer's instructions following programs for polymerase chain reaction (PCR): initial denaturation at 94 °C for 2 min followed by 30 cycles of denaturation of 98 °C for 10 s, annealing at 62 °C for the 30s, polymerization at 68 °C for 1 min, and final elongation at 68 °C for 7 min. Five microliters of each amplification mixture were verified by agarose (1% w/v) gel electrophoresis in 0.5X Tris-borate-EDTA (TBE) buffer. The partial sequences were generated using the following ITS4 and ITS5 primers from a company (1st BASE Company, Malaysia).

Phylogenetic analysis

The PCR amplified products were purified using a

commercial kit and then incubated at 37 °C for 60 min followed by 80 °C for 20 min. The nucleotide sequences were determined using dideoxy sequencing techniques at 1st BASE Company, Malaysia (taken as commercial service). Partial sequences were generated using the ITS4 and ITS5 primers. The ITS sequences were combined using the Bioedit software, checked manually, corrected, and then analyzed using the Basic Local Alignment Search Tool (BLAST) available on the National Center for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/) to search for nucleotide sequence homology in GenBank. Phylogenetic analyses were conducted using the using MEGA version 6.06 program [30,31] and a neighbor-joining tree was constructed using the Kimura two-parameter model. The phylogenetic tree was generated using the most identical fungal sequences available in the GenBank database. Confidence values were assessed from 1,000 bootstrap replicates of the original data.

Results and discussion

Collection and cultural and morphological characterization of isolates of *S. sclerotiorum*

A total of one hundred and eighty isolates of *S. sclerotiorum* were excised from infected samples collected from different districts namely Rangpur, Dinajpur, Panchagarh, Lalmonirhat, Jessore, Sirajganj, Jamalpur, Mymensingh, Tangail, Pabna, Natore and Bogra of Bangladesh during 2016-17 and 2017-18 cropping years. The samples were collected from different host plants viz. mustard, marigold, bush bean, garden pea, broccoli, country bean, and ornamental gourd based on the symptom developed by the pathogen (Figure 1).

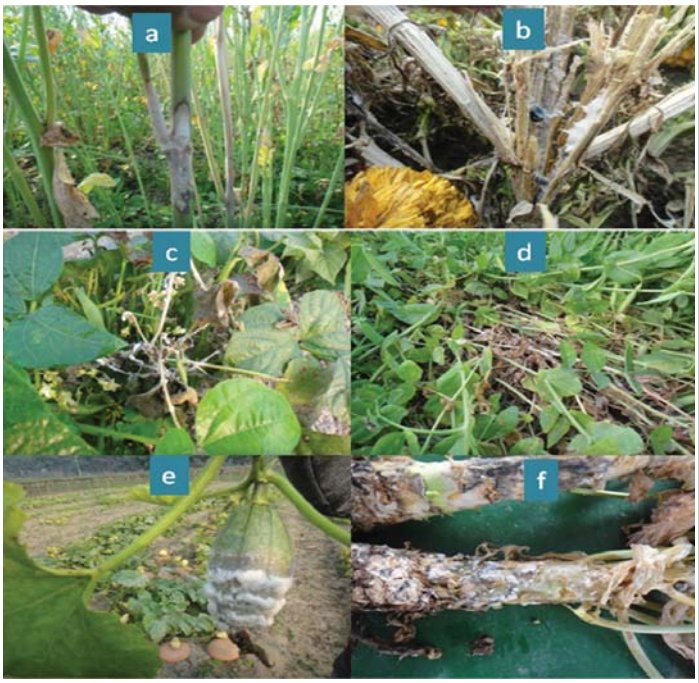

Figure 1: Common symptoms of white mold disease (a) mustard (b) marigold (c) bush bean (d) garden pea (e) ornamental gourd and (f) broccoli caused by *Sclerotinia sclerotiorum*; observing sclerotia inside marigold plant and infected plants of bush bean and broccoli.

All the one hundred and eighty isolates of *S. sclerotiorum* were found to be variable to some extent in colony colour, and type of growth based on cultural characteristics of mycelium (Table 1). Few isolates showed dirty white colony colour, while the rest of the isolates showed whitish colony colour which is predominant among the isolates (Table 1 and Figure 2). Similar data were obtained by Grabicoski, [32] when evaluating 57 isolates of *S. sclerotiorum* and verified the

Table 1: Cultural variability of *Sclerotinia sclerotiorum* isolates collected from several host in different regions of Bangladesh.

Code of isolates	Cultural variability		
	Colony colour	Type of growth	Av. Mycelia growth at 72 hrs (cm)
SS1	Whitish	Fluffy and regular	7.50
SS2	Dirty white	Sparse and regular	7.80
SS3	Whitish	Fluffy and irregular	5.60
SS4	Whitish	Fluffy and regular	4.60
SS5	Whitish	Sparse and regular	5.30
SS6	Whitish	Fluffy and regular	6.30
SS7	Dirty white	Sparse and regular	5.70
SS8	Whitish	Fluffy and irregular	8.10
SS9	Whitish	Fluffy and regular	8.00
SS10	Whitish	Fluffy and irregular	8.30
SS11	Whitish	Fluffy and irregular	6.40
SS12	Whitish	Sparse and regular	4.80
SS13	Dirty white	Sparse and irregular	3.90
SS14	Dirty white	Sparse and regular	3.80
SS15	Dirty white	Fluffy and regular	4.60
SS16	Dirty white	Sparse and regular	4.30
SS17	Whitish	Fluffy and regular	3.80
SS18	Whitish	Fluffy and regular	4.30
SS19	Whitish	Sparse and irregular	4.10
SS20	Whitish	Sparse and irregular	4.10
SS21	Dirty white	Sparse and irregular	6.10
SS22	Dirty white	Sparse and regular	6.30
SS23	Whitish	Sparse and regular	4.65
SS24	Whitish	Fluffy and regular	5.35
SS25	Dirty white	Sparse and regular	8.00
SS26	Dirty white	Fluffy and regular	4.60
SS27	Whitish	Sparse and irregular	7.60
SS28	Whitish	Fluffy and irregular	3.85
SS29	Whitish	Fluffy and irregular	5.72
SS30	Dirty white	Sparse and regular	5.00
SS31	Whitish	Fluffy and irregular	8.00
SS32	Whitish	Fluffy and regular	5.50
SS33	Whitish	Sparse and regular	5.30
SS34	Whitish	Fluffy and regular	4.10
SS35	Dirty white	Sparse and regular	6.27
SS36	Whitish	Fluffy and regular	6.67
SS37	Whitish	Fluffy and regular	7.25
SS38	Whitish	Fluffy and irregular	7.15
SS39	Whitish	Fluffy and irregular	4.16
SS40	Whitish	Sparse and regular	4.35
SS41	Dirty white	Sparse and irregular	4.72
SS42	Dirty white	Sparse and regular	3.70
SS43	Dirty white	Fluffy and regular	3.65
SS44	Whitish	Sparse and regular	2.85
SS45	Whitish	Fluffy and regular	7.05
SS46	Whitish	Fluffy and regular	6.85
SS47	Whitish	Fluffy and regular	6.63
SS48	Whitish	Sparse and regular	6.02
SS49	Dirty white	Fluffy and regular	6.85
SS50	Dirty white	Sparse and irregular	4.85
SS51	Whitish	Fluffy and irregular	8.15

SS52	Dirty white	Fluffy and irregular	6.50
SS53	Dirty white	Sparse and regular	5.62
SS54	Whitish	Fluffy and irregular	6.82
SS55	Whitish	Sparse and regular	5.45
SS56	Whitish	Fluffy and regular	6.25
SS57	Dirty white	Sparse and irregular	5.15
SS58	Whitish	Fluffy and regular	6.15
SS59	Dirty white	Sparse and regular	5.25
SS60	Whitish	Fluffy and irregular	5.40
SS61	Whitish	Fluffy and regular	4.15
SS62	Whitish	Sparse and regular	6.77
SS63	Whitish	Fluffy and regular	5.15
SS64	Dirty white	Sparse and regular	6.15
SS65	Whitish	Fluffy and regular	7.15
SS66	Whitish	Sparse and irregular	5.15
SS67	Whitish	Fluffy and irregular	2.65
SS68	Whitish	Fluffy and irregular	6.15
SS69	Whitish	Sparse and regular	5.12
SS70	Dirty white	Sparse and irregular	7.50
SS71	Dirty white	Sparse and regular	7.15
SS72	Dirty white	Fluffy and regular	6.22
SS73	Whitish	Sparse and regular	6.17
SS74	Dirty white	Fluffy and regular	5.20
SS75	Whitish	Fluffy and regular	4.25
SS76	Whitish	Fluffy and regular	7.10
SS77	Whitish	Fluffy and regular	6.10
SS78	Dirty white	Sparse and irregular	6.05
SS79	Whitish	Sparse and regular	5.18
SS80	Dirty white	Sparse and regular	6.20
SS81	Dirty white	Sparse and regular	6.12
SS82	Dirty white	Fluffy and regular	4.17
SS83	Whitish	Sparse and regular	6.25
SS84	Dirty white	Fluffy and irregular	4.82
SS85	Whitish	Fluffy and regular	3.95
SS86	Whitish	Sparse and regular	6.18
SS86	Whitish	Fluffy and regular	6.18
SS87	Whitish	Sparse and regular	5.42
SS88	Dirty white	Fluffy and irregular	5.40
SS89	Whitish	Fluffy and regular	6.25
SS90	Whitish	Fluffy and irregular	6.36
SS91	Whitish	Fluffy and irregular	5.37
SS92	Whitish	Sparse and regular	5.15
SS93	Whitish	Sparse and irregular	5.22
SS94	Dirty white	Sparse and regular	5.10
SS95	Dirty white	Fluffy and regular	6.15
SS96	Dirty white	Sparse and regular	6.20
SS97	Whitish	Fluffy and regular	6.22
SS98	Dirty white	Fluffy and regular	4.53
SS99	Whitish	Sparse and irregular	7.17
SS100	Whitish	Sparse and irregular	7.47
SS101	Whitish	Sparse and irregular	7.60
SS102	Dirty white	Sparse and regular	7.75
SS103	Dirty white	Sparse and irregular	7.35
SS104	Whitish	Sparse and irregular	5.72
SS105	Whitish	Sparse and regular	7.30
SS106	Whitish	Sparse and irregular	6.97
SS107	Whitish	Sparse and irregular	5.67
SS108	Dirty white	Sparse and regular	6.85
SS109	Whitish	Fluffy and regular	5.60
SS110	Whitish	Sparse and regular	7.47
SS111	Whitish	Fluffy and irregular	6.90
SS112	Whitish	Fluffy and regular	4.95
SS113	Whitish	Sparse and regular	5.40
SS114	Dirty white	Fluffy and regular	6.27
SS115	Dirty white	Sparse and regular	6.10
SS116	Dirty white	Fluffy and regular	4.07

SS117	Whitish	Fluffy and regular	6.65
SS118	Dirty white	Fluffy and irregular	4.15
SS119	Whitish	Fluffy and irregular	5.90
SS120	Whitish	Sparse and regular	6.75
SS121	Whitish	Fluffy and regular	7.35
SS122	Whitish	Sparse and regular	6.40
SS123	Whitish	Fluffy and irregular	4.80
SS124	Dirty white	Fluffy and regular	7.15
SS125	Dirty white	Sparse and regular	6.35
SS126	Dirty white	Sparse and irregular	4.85
SS127	Whitish	Sparse and irregular	5.25
SS128	Whitish	Sparse and regular	6.20
SS129	Dirty white	Fluffy and regular	4.85
SS130	Dirty white	Fluffy and irregular	6.25
SS131	Whitish	Fluffy and irregular	5.15
SS132	Whitish	Sparse and regular	3.95
SS133	Whitish	Sparse and irregular	5.15
SS134	Dirty white	Sparse and regular	5.18
SS135	Dirty white	Fluffy and regular	4.85
SS136	Whitish	Sparse and regular	2.95
SS137	Whitish	Fluffy and regular	6.16
SS138	Whitish	Fluffy and regular	4.85
SS139	Whitish	Sparse and irregular	5.45
SS140	Dirty white	Sparse and irregular	4.16
SS141	Whitish	Sparse and irregular	5.15
SS142	Whitish	Sparse and regular	6.85
SS143	Whitish	Sparse and regular	7.02
SS144	Whitish	Sparse and regular	7.72
SS145	Whitish	Sparse and regular	4.95
SS146	Dirty white	Sparse and irregular	4.85
SS147	Dirty white	Sparse and irregular	6.15
SS148	Dirty white	Sparse and regular	4.95
SS149	Whitish	Fluffy and regular	6.25
SS150	Whitish	Sparse and regular	6.16
SS151	Whitish	Fluffy and irregular	7.10
SS152	Whitish	Fluffy and regular	7.15
SS153	Whitish	Sparse and regular	4.93
SS154	Dirty white	Fluffy and regular	5.70
SS155	Dirty white	Sparse and regular	6.85
SS156	Whitish	Fluffy and regular	6.62
SS157	Whitish	Fluffy and regular	7.50
SS158	Dirty white	Fluffy and irregular	6.85
SS159	Whitish	Fluffy and irregular	5.72
SS160	Dirty white	Sparse and regular	5.80
SS161	Whitish	Fluffy and regular	4.85
SS162	Dirty white	Sparse and regular	5.15
SS163	Whitish	Fluffy and irregular	4.10
SS164	Dirty white	Fluffy and regular	4.18
SS165	Whitish	Sparse and regular	6.26
SS166	Whitish	Fluffy and regular	4.80
SS167	Whitish	Sparse and regular	4.95
SS168	Whitish	Fluffy and irregular	6.12
SS169	Dirty white	Fluffy and regular	5.00
SS170	Whitish	Fluffy and irregular	4.67
SS171	Whitish	Fluffy and irregular	3.80
SS172	Whitish	Sparse and regular	6.35
SS173	Whitish	Sparse and irregular	3.40
SS174	Whitish	Sparse and regular	6.42
SS175	Dirty white	Fluffy and regular	4.85
SS176	Whitis	Sparse and regular	6.65
SS177	Dirty white	Fluffy and regular	6.90
SS178	Dirty white	Fluffy and regular	2.85
SS179	Whitish	Sparse and irregular	7.35
SS180	Whitish	Sparse and irregular	7.80

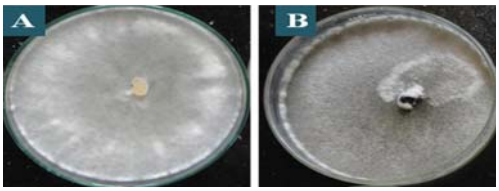

Figure 2: Predominant staining in *S. sclerotiorum* colonies in PDA culture medium, after 7 days of incubation, being: A: whitish colony which is predominant among the isolates, B: dirty white colony.

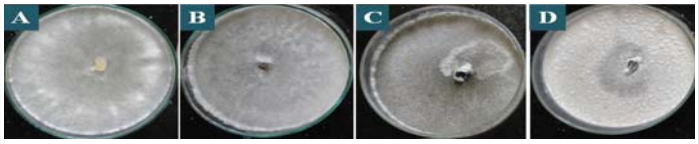

Figure 3: Mycelial types in colonies of *S. sclerotiorum* on PDA culture medium, after 7 days of incubation, being: A: fluffy mycelia with regular, B: fluffy mycelia with irregular, C: sparse mycelia with regular, D: sparse mycelia with irregular type of growth.

predominance is white mycelium in *S. sclerotiorum* cultured in a BPD medium. Sharma, et al. [33] found differences in colony colour among the isolates collected from the different hosts as whitish and dirty white, however, white and grey white colony colour as observed by them were not found in any of the isolates in the present study. However, Ziman, et al. [34] observed a slight variation in colony colour of *S. sclerotiorum* isolates collected from different hosts, which differentiate from white to brown but the white colour was predominant in most of the isolates. The variations in the type of mycelia growth were also observed. The *S. sclerotiorum* isolates showed fluffy and sparse mycelia with the regular and irregular types of growth (Table 1 and Figure 3). Basha & Chatterjee, [35] also observed variation in the type of mycelial growth as colonies of seventeen isolates were fluffy, whereas three showed compact mycelia. Choudhary and Prasad, [36] also observed two types of mycelia growth as fluffy and compact among different isolates. However, Sharma, et al. [33] observed three types of scattered, smooth, and fluffy mycelia growth among different isolates.

The mycelia growth rate of *S. sclerotiorum* differed considerably among the isolates (Table 1). The average mycelial growth ranges from 2.65 cm to 8.10 cm at 72 hrs after inoculation was recorded. According to the mycelia growth at 72 hrs after inoculation, all the isolates were divided into three groups' viz. slow-growing isolates (average mycelial growth as colony diameter 0.0-4.0 cm at 72 hrs after inoculation), intermediated growing isolates (average mycelial growth as colony diameter 4.10-6.99 cm at72 hrs after inoculation) and fast-growing isolates (average mycelial growth as colony diameter ≥7.00 cm at72 hrs after inoculation). In the present study, the *S. sclerotiorum* isolates SS67, SS44, SS178, SS136, SS171, SS43, SS42, SS14, SS17, SS171, SS28, SS13, SS85 and SS132 isolates showed slow mycelia growth as colony diameter was 2.65, 2.85, 2.85, 2.95, 3.40, 3.65, 3.70, 3.80, 3.80, 3.80, 3.85, 3.90, 3.95 and 3.95 cm after 72 hrs of incubation, respectively, while the isolates SS143, SS45, SS76, SS 151, SS38, SS65, SS71, SS124, SS152, SS99, SS37, SS105, SS103, SS121, SS179, SS100, SS110, SS1, SS 70, SS157, SS27, SS101, SS144, SS102, SS2, SS180, SS9, SS25, SS31, SS8, SS51, SS10 and SS36 showed fast mycelia growth with colony diameter of 7.02, 7.05, 7.10, 7.10, 7.15, 7.15, 7.15, 7.15, 7.15, 7.17, 7.25, 7.30, 7.35, 7.35, 7.35, 7.47, 7.47, 7.50, 7.50, 7.50, 7.60, 7.60, 7.72, 7.75, 7.80, 7.80, 8.00, 8.00, 8.00, 8.10, 8.15 8.30 and 8.67 cm after 72 hrs of incubation, respectively (Table 1). The rest of the collected isolates of *S. sclerotiorum* showed intermediate mycelia growth with colony diameter from 4.01 to 6.99 cm after 72 hrs of incubation. However, all intermediated and fast-growing isolates of *S. sclerotiorum* covered full mycelia growth in the 90 mm diameter Petri plates within 96 h of incubation while the slow-growing isolates of *S. sclerotiorum* covered full mycelia growth in the 90 mm diameter Petri plates after 120 h of incubation. A similar trend was also reported by Corradini, [17] who evaluated 19 isolates of *S. sclerotiorum* and observed that the colonies reached the maximum diameter of the plaque at the end of 120 hrs of incubations. Garg, et al. [37] reported significant differences between isolates with the colony diameter measured after 24 and 48 h of incubation. Ahmadi, et al. [38] examined seven populations of *S. sclerotiorum* associated with stem rot of important crops and weeds and based on mycelia growth; these seven populations were classified into four groups i.e. very fast, fast, intermediate and slow-growing.

All the isolates presented sclerotia production (Table 2). The size, shape, and number, pattern of sclerotia formation varied among the isolates (Figure 4 and Table 2). Four different patterns of sclerotia formation viz. near to rim of the plaque, attached to the rim of the plaque, scattered all around the plaque and ring centre of the plaque, were observed among the isolates were near to the rim is predominant (Figure 4 and Table 2). Similar data were found by Zanatta, et al. [39] who reported that the distribution of sclerotia was 60% near the margin of the plaque, 25% scattered in the plaque, and 15% concentric circle in the plaque. As the shape of the sclerodes formed, 34.44% of the isolates presented a rounded shape and 65.56% irregular shape. These data corroborate those of Grabicoski, [32] who classified most of the isolates (65%) produced as diverse, with varied formats of sclerodes.

Regarding the time required for the formation of the first sclerotia of each isolate, there was a distinct difference among the isolates. The time for sclerotia formation varied from 5.00 days to 15.00 days. Similar data were found by Grabicoski, [32], and the meantime for sclerotia formation ranged from 11.8 to 15.4 days. For Abreu, [40], the time for the formation

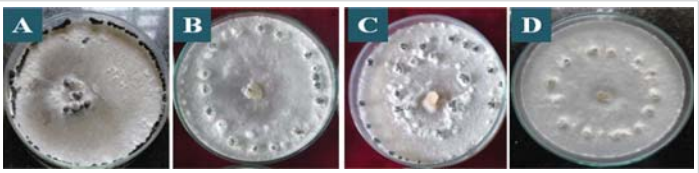

Figure 4: Distribution of sclerotia on the plaques of *S. sclerotiorum* colonies in PDA culture medium, after 20 days of incubation, being: A: attached to rim of the plaque, B: near to rim of the plaque, C: scattered all around of the plaque, D: ring centre of the plaque.

Table 2: Morphological variability of Sclerotinia sclerotiorum isolates collected from several host in different regions of Bangladesh.

	Sclerotia formation		
Code of isolates	Initiation (DAI)	Average number sclerotia plate^{-1}	Pattern of sclerotia production
SS1	5	53.50	Attached to rim
SS2	5	52.00	Near to rim
SS3	7	36.50	Near to rim
SS4	8	22.00	Double ring near to rim and centre
SS5	8	24.00	Scattered all around
SS6	6	50.00	Near to rim
SS7	8	31.00	Scattered all around
SS8	6	56.50	Near to rim
SS9	7	47.50	Attached to rim
SS10	5	47.50	Near to rim
SS11	8	22.00	Double ring near to rim and centre
SS12	9	24.50	Near to rim
SS13	8	20.00	Scattered all around
SS14	13	11.50	Near to rim
SS15	8	23.50	Near to rim
SS16	8	20.50	Near to rim
SS17	12	20.50	Scattered all around
SS18	11	21.50	Near to rim
SS19	12	18.50	Near to rim
SS20	7	28.00	Near to rim
SS21	7	31.00	Scattered all around
SS22	6	43.50	Attached to rim
SS23	10	20.00	Scattered all around
SS24	9	28.00	Attached to rim
SS25	8	25.50	Near to rim
SS25	9	25.50	Near to rim
SS26	13	15.00	Near to rim
SS27	9	42.00	Scattered all around
SS28	5	56.50	Near to rim
SS29	6	41.00	Scattered all around
SS30	9	35.00	Near to rim
SS31	5	52.00	Attached to rim
SS32	12	19.00	Near to rim
SS33	9	40.00	Double ring near to rim and centre
SS34	8	42.50	Double ring near to rim and centre
SS35	5	56.00	Near to rim
SS36	10	35.00	Attached to rim
SS37	13	18.50	Near to rim
SS38	8	42.00	Attached to rim
SS39	12	19.00	Near to rim
SS40	10	27.00	Scattered all around
SS41	13	15.00	Scattered all around
SS42	9	32.00	Attached to rim
SS43	8	10.00	Near to rim
SS44	8	10.00	Near to rim
SS45	10	25.00	Near to rim
SS46	8	35.00	Scattered all around
SS47	9	28.00	Near to rim
SS48	6	43.00	Attached to rim
SS49	7	35.50	Near to rim
SS50	11	17.00	Scattered all around
SS51	12	34.50	Near to rim
SS52	11	23.50	Double ring near to rim and centre
SS53	10	30.00	Near to rim
SS54	6	53.50	Scattered all around
SS55	7	40.00	Double ring near to rim and centre
SS56	11	30.00	Scattered all around
SS57	13	19.50	Near to rim
SS58	11	36.00	Near to rim
SS59	10	30.00	Scattered all around
SS60	8	37.50	Attached to rim
SS61	12	25.00	Scattered all around
SS62	13	23.00	Attached to rim
SS63	10	26.00	Near to rim
SS64	11	27.00	Near to rim
SS65	7	41.00	Scattered all around
SS66	12	35.00	Near to rim
SS67	13	18.50	Scattered all around
SS68	8	32.50	Near to rim
SS69	11	28.50	Scattered all around
SS70	5	60.00	Double ring near to rim and centre
SS71	7	43.00	Near to rim
SS72	12	29.50	Double ring near to rim and centre
SS73	13	20.50	Scattered all around
SS74	13	22.50	Scattered all around
SS75	12	21.00	Near to rim
SS76	7	42.50	Near to rim
SS77	12	18.00	Near to rim
SS78	11	25.00	Scattered all around
SS79	10	30.50	Attached to rim
SS80	7	45.00	Scattered all around
SS81	5	51.00	Attached to rim
SS82	8	24.00	Near to rim
SS83	6	47.50	Near to rim
SS84	5	40.00	Near to rim
SS85	13	24.00	Scattered all around
SS86	12	33.00	Attached to rim
SS86	12	27.00	Scattered all around
SS87	7	46.00	Attached to rim
SS88	10	28.00	Near to rim
SS89	10	32.50	Near to rim
SS90	6	51.00	Near to rim
SS91	9	35.00	Attached to rim
SS92	8	31.00	Near to rim
SS93	12	25.00	Scattered all around
SS94	13	21.00	Near to rim
SS95	12	32.00	Double ring near to rim and centre
SS96	9	30.00	Double ring near to rim and centre
SS97	6	58.00	Attached to rim
SS98	7	34.50	Double ring near to rim and centre
SS99	7	48.00	Near to rim
SS100	5	64.00	Attached to rim
SS101	8	35.00	Near to rim
SS102	5	59.00	Attached to rim
SS103	8	40.00	Near to rim
SS104	11	33.00	Scattered all around
SS105	8	41.00	Attached to rim
SS106	5	57.50	Near to rim
SS107	7	58.50	Attached to rim
SS108	9	38.50	Near to rim
SS109	7	49.50	Near to rim
SS110	8	42.50	Scattered all around
SS111	9	35.00	Near to rim
SS112	9	43.50	Scattered all around
SS113	8	41.50	Near to rim
SS114	7	44.00	Scattered all around
SS115	6	56.00	Near to rim
SS116	7	51.00	Double ring near to rim and centre
SS117	13	21.50	Double ring near to rim and centre
SS118	13	14.00	Scattered all around
SS119	11	22.50	Attached to rim
SS120	12	20.00	Near to rim
SS121	13	18.00	Scattered all around
SS122	11	29.50	Near to rim
SS123	12	20.00	Scattered all around
SS124	9	35.50	Near to rim
SS125	6	55.00	Attached to rim

SS126	10	28.50	Near to rim
SS127	9	29.50	Scattered all around
SS128	12	20.00	Scattered all around
SS129	8	29.00	Near to rim
SS130	6	42.50	Attached to rim
SS131	13	24.00	Near to rim
SS132	13	18.00	Near to rim
SS133	12	26.00	Scattered all around
SS134	11	17.50	Near to rim
SS135	7	30.00	Double ring near to rim and centre
SS136	15	9.00	Near to rim
SS137	14	34.00	Near to rim
SS138	15	16.00	Scattered all around
SS139	12	28.50	Near to rim
SS140	15	16.00	Near to rim
SS141	14	30.00	Near to rim
SS142	8	38.00	Double ring near to rim and centre
SS143	7	45.00	Attached to rim
SS144	15	17.00	Scattered all around
SS145	14	17.50	Attached to rim
SS146	15	22.50	Near to rim
SS147	11	35.00	Double ring near to rim and centre
SS148	12	29.00	Scattered all around
SS149	9	30.00	Near to rim
SS150	14	16.50	Scattered all around
SS151	6	52.00	Near to rim
SS152	14	19.00	Scattered all around
SS153	8	40.00	Near to rim
SS154	7	42.50	Double ring near to rim and centre
SS155	7	56.00	Attached to rim
SS156	13	35.00	Near to rim
SS157	14	18.50	Near to rim
SS158	6	42.00	Near to rim
SS159	14	19.00	Scattered all around
SS160	12	27.00	Near to rim
SS161	12	27.00	Scattered all around
SS162	13	15.00	Scattered all around
SS163	14	32.00	Attached to rim
SS164	15	10.00	Near to rim
SS165	15	10.00	Near to rim
SS166	14	25.00	Near to rim
SS167	8	35.00	Scattered all around
SS168	12	28.00	Near to rim
SS169	8	43.00	Scattered all around
SS170	9	35.50	Near to rim
SS171	13	17.00	Scattered all around
SS172	8	34.50	Near to rim
SS173	12	23.50	Double ring near to rim and centre
SS174	13	30.00	Double ring near to rim and centre
SS175	6	53.50	Attached to rim
SS176	8	40.00	Attached to rim
SS177	12	30.00	Near to rim
SS178	14	19.50	Near to rim
SS179	12	36.00	Near to rim
SS180	12	30.00	Near to rim

of sclerodium in the isolates evaluated ranged from 4.00 to 12.44 days. Zanatta, et al. [39] and reported that the time for sclerotia formation varied from 10.67 to 18.0 days.

The number of sclerotia per plaque of different isolates varied considerably and the range from 9.00 to 64.00 sclerotia was produced per plaque (Table 2). The *S. sclerotiorum* isolates SS136, SS43, SS44, SS14, SS118, SS26, SS 41, SS138, SS140, SS 150, SS 50, SS144, SS170, SS134 , SS145, SS77, SS121, SS132, SS157, SS19, SS 37, SS67, SS32, SS39, SS152, SS159, SS57, SS177, SS23, SS120, SS123 and SS128 were showed lower average number of sclerotia production per plaque range from 9.00 to 20.00 sclerotia per plaque, where isolates SS80, SS143, SS87, SS9, SS10, SS83, SS99, SS109, SS6, SS81, SS90, SS116, SS2, SS31, SS151, SS1, SS54, SS175, SS125, SS35, SS115, SS155, SS8, SS28, SS106, SS97, SS107, SS102, SS70 and SS100 showed higher average number of Sclerotia per plate range from 45.00 to 64.00 sclerotia per plaque (Table 2). The data found in the present study corroborating with studies developed by Abreu, [40], and the number of sclerodes ranged from 10.33 to 46.00, and by Grabicoski, [32], ranging from 16.6 to 57.2 sclerotia per plaque. Ghasolia and Shivpuri, [41] also observed variability among 38 isolates of *S. sclerotiorum*, which showed variation in their morphological traits like a sclerotial number, size, position, and pattern. Kumar, et al. [26] also examined sufficient diversity in size of sclerotia and pattern of sclerotia among isolates *S. sclerotiorum.* As to the shape of the sclerotia formed, only 37.78% of isolates presented a rounded shape (68 isolates) and the rest of the isolates (63.22%) presented sclerotia with a different format (112 isolates) (Figure 5). These data corroborated those of Grabicoski, [32] who classified most of the isolates (65%) as diverse, with varied formats. Zanatta, et al. [39] reported three different shapes of sclerotia formed in *S. sclerotiorum,* 25% rounded shape, 30% irregular shape, and 45% in a different format.

Molecular characterization and genetic diversity of *S. sclerotiorum*

A total of 14 samples were selected for DAN extraction (Figure 6). After DNA extraction, the DNA was used for PCR using ITS primers ITS4 (TCCTCCGCTTATTGATATGC) and ITS5 (GGAAGTAAAAGTCGTAACAAGG) for amplification ITS regions. During PCR all the DNA samples of the isolates were amplified properly and those were verified by agarose gel electrophoresis (Figure 7). Amplified DNA was sent for sequencing for

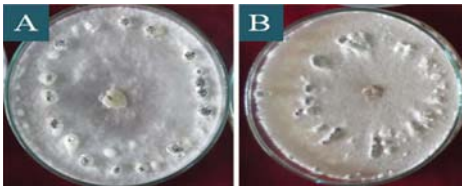

Figure 5: Format of the sclerotia formed on the plaques of *S. sclerotiorum* colonies in PDA culture medium, after 20 days of incubation, being: A: rounded shaped, B: divers format.

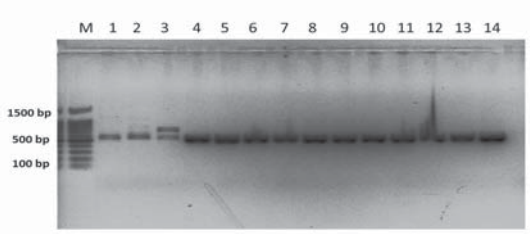

Figure 6: DNA amplification profile of fourteen *S. sclerotiorum* isolates with ITS4 forward and ITS5 reverse primer. M-50 bp ladder.

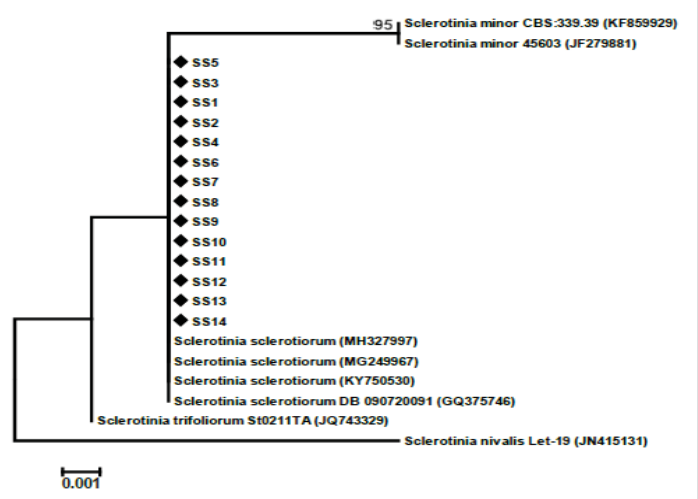

Figure 7: Phylogenetic tree based on internal transcribed spacer sequences revealing the phylogenetic relationships among the *Sclerotinia sclerotiorum* isolates and related species. The 14 isolates from this study are indicated in the tree with a black diamond.

molecular characterization. Molecular characterization of the 14 isolates by ITS sequencing indicated all the tested isolates were identified as *Sclerotinia sclerotiorum*. The ITS sequences of the 14 isolates were identical to many publicly available *S. sclerotiorum* sequences (eg. KY750530). Phylogenetic analysis of the isolates based on ITS sequences revealed the isolates belonged to a similar group of publicly available *S. sclerotiorum* and were dissimilar from the group of *S. minor, S. trifolium,* and distinctly different from the *S. nivalis* group (Figure 4).

Conclusion

The evaluated populations presented wide variability for the cultural and morphological characteristics, being predominant in the whitish colonies. The majority of isolates produced a higher number of sclerotia near the margin of the plates and with diverse formats. Phylogenetic analysis revealed that the isolates belonged to a similar group of publicly available *S. sclerotiorum* and were dissimilar from the group of *S. minor,* and *S. trifolium* and distinctly differ from *S. nivalis* group. The present study is the first evidence for morphological and genetic diversity study of *S. sclerotiorum* in Bangladesh. Therefore, this report contributes to more information about the epidemiology of the disease and can be useful in implementing effective management strategies for the disease.

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