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(RESEARCH ARTICLE)



## Molecular characterization of the pathogen responsible for *Choanephora* fruit rot disease in *Momordica charantia* (L.) and establishment of its ecofriendly control measures

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### Abstract

The present investigation was carried out to characterize the pathogen responsible for *Choanephora* fruit rot disease in bitter melon along with find out appropriate control measure using biological resources. The pathogenic fungal strain was isolated from the disease infected fruit part of bitter melon. It was subjected to four different types of growth media named potato dextrose agar (PDA), Czapeck Dox agar (CDA), nutrient agar (NA) and Sabouraud dextrose agar (SDA) were used to note down the differences regarding to various characteristics appeared due to dissimilation of culture medium. Thereafter, the growth environment and medium contents were changed to evaluate the effect of temperature, pH, carbohydrate, sugar concentration, salt, organic acid etc. on the mycelial growth of the concerned fungus. In the experiment, these parameters significantly influenced the growth pattern of the fungus. For proper identification of the fungus, advanced molecular technique was used. The PCR product of the internal transcribed spacer (ITS) region of the fungi showed nearly 650 bp size of clear band followed by sequencing of the PCR product of the fungus. The sequenced data showed 99% similarities with the original sequence of *Choanephora cucurbitarum*. In search of suitable biocontrol measure, the antifungal activities of the seven plant extracts namely *Hibiscus rosa-sinensis*, *Ficus racemosa*, *Moringa oleifera*, *Psidium guajava*, *Azadirachta indica*, *Cassia alata* and *Coccinia grandis* were assayed where *Azadirachta indica* (42.12%) and *Moringa oleifera* (35.71%) had promising inhibitory effects against the isolated fungus. The outcome of the experiment would be benevolent for the detection and development of eco-friendly control approach of the detrimental disease of the bitter melon.

**Keywords:** *Momordica charantia*; *Choanephora* fruit rot disease; Characterizations; Bio-control

### 1. Introduction

The Cucurbitaceae is a medium sized family of dicotyledons consisting of an excellent example of a plant family with many economically useful species [1]. Though many species of the family are cultivated for edible purposes [2], they are also known to contain many bioactive compounds such as cucurbitacins, triterpenes, sterols and alkaloids [3] that have anti-inflammatory, antitumor, hepato-protective, cardiovascular and immune-regulatory activities [4-5]. *Momordica charantia* (L.), bitter melon one of the most important member of *Cucurbitaceae* family, is a flowering vine that widely cultivated in Asia, East Africa, and South America [6] for its intensely bitter fruits that are commonly used in cooking

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and as healer [7] for treating various diseases [8]. It is a climbing perennial that usually grows up to 5 meters, and bears elongated fruits with a knobby surface. Bitter melon is one of the most popular vegetable in Asia where it is grown on approximately 340,000 hectare annually by smallholder farmers [9]. In Bangladesh, the commercial cultivation of bitter melon is very successful which covered around 23,890 acres of cultivable lands and the total production is nearly 52,020 metric tons per annum [10]. The success of any crop production generally depends on the profitability at farm level. Bitter melon producing farmers often fail to obtain the expected yield due to the infestation of the crops by insect-pests and diseases and non-adoption of plant protection measures by farmers [11]. According to Muthuraman & Kumar [12] report, the losses ranging from 20-30% are resulted from each of the insect-pests, diseases and weeds. Among the various diseases inflicting bitter melon, *Choanephora* fruit rot is one of the most devastating diseases that also affect other cucurbits [13]. The disease is caused by the fungus *Choanephora cucurbitarum* that is favored by extended period of warm and wet weather and the symptom of the disease includes soft, rotted blossom end covered with fluffy purplish black fungal growth. The catastrophic loss caused by the diseases implies that suitable control measures must be followed to keep these losses to the minimum [12]. But the intensive and indiscriminate use of pesticides in agriculture has caused many problems to the environment and responsible for food contamination, poisoning of farmers, and elimination of non-target organisms [14]. On the other hand, plants have ability to synthesize aromatic secondary metabolites, like phenols, phenolic acids, quinones, flavones, flavonoids, flavonols, tannins and coumarins [15]. These groups of compounds show antimicrobial effect and may take part in plant defense mechanisms against pathogenic microorganisms [16]. The present study was purposed to evaluate the antifungal potential of some plant extracts against the phyto-pathogenic fungi *Choanephora cucurbitarum* along with its identification and characterization.

## 2. Material and methods

### 2.1. Collection of diseased sample

The disease infected immature fruits (figure 1) was collected into closed paper envelop [17] from the field of bitter melon in Naogaon, Rajshahi, Bangladesh and the disease was identified by Dr. Asrafal Islam, Scientific Officer, Fruit Research Institute, Binodpur, Rajshahi. The experiment related to the disease and causal agent was conducted at Professor Joarder DNA and Chromosome Research Lab., Department of Genetic Engineering and Biotechnology, University of Rajshahi, Bangladesh.



**Figure 1** Showing the disease infected fruit sample of bitter melon

### 2.2. Isolation of the pathogen responsible for the disease

At the laboratory, the infected fruit tissues were excised with a sterilized razor blade at the point of progression of disease symptom; then surface sterilized by 70% ethanol solution for 2 minutes. The tissues were then washed with sterilized distilled water three times and were placed on sterile paper towels for drying [18], followed by incubation on PDA (Hi-Media, India) at 25 °C in the dark for 3 days. The medium was supplemented with 0.5 gL<sup>-1</sup> of streptomycin sulphate (Sigma-Aldrich, USA) and 1mL<sup>-1</sup> of lactic acid. The mycelium emerged from diseased sample were re-isolated using a single spore technique [19] and transferred onto the Petri plate of new fresh PDA medium for the incubation of 7 days.

### 2.3. Morphological and microscopic identification

The isolated fungi were identified according to colony morphology and microscopic examination. Fungal colonies were grown onto PDA medium for species identification. The purified fungi were identified according to the fungal morphological characteristics as described in several previous studies [20-22]. The characteristics such as diameters,

overall colors, colors of conidia, texture, form, margin, reverse colors, elevation, opacity, dry weight, sporulation, and zonation were studied.

#### 2.4. Growth profiling of the fungal isolate

In general, various artificially made growth medium are used for the study of fungi that influence the mycelial growth and colony morphology of the concerned fungi [23].

In our study, PDA (Hi-Media, India), CDA (Hi-Media, India), NA (Hi-Media, India) and SDA (Hi-Media, India) media were used for observation of various characteristics of the fungal colonies in response to different culture medium. After incubation of 7 days in dark, different morphological characteristics of the fungal colonies such as form, margin, elevation, surface, opacity, front colour, back colour and dry weight were observed [24]. To determine the effect of temperature on the growth of the fungal strain, the fungal Petri plates were kept in 15 °C, 20 °C, 25 °C, 30 °C and 35 °C temperature, respectively. Thereafter, the carbohydrates dextrose, fructose, lactose, maltose and starch were used as sole carbon source in modified PDA media to check the effect of carbohydrates on the growth of fungi. For the growth profiling in different concentrations of sugar, 3%, 6%, 9%, 12%, 15% and 18% of dextrose were added in PDA media instead of 2% dextrose. To study about the effect of salts on the mycelia of the strain, 5% of different salts named CaCl<sub>2</sub>, MgCl<sub>2</sub>, MgSO<sub>4</sub>, NaCl and KCl were included in the PDA medium. The effect of organic acids on the growth of fungi was also estimated by adding 1% of citric acid, malic acid and nicotinic acid separately in PDA medium and incubating it at dark for 7 days.

#### 2.5. Molecular identification

Identifying fungi based on morphology alone may be challenging, and this practice is no longer acceptable according to the latest International Code of Nomenclature for algae, fungi, and plants [25-26]. As a consequence, DNA sequence-based methods have been widely used for identifying species within the mega diverse fungi [27-30]. According to Schoch et al., [31] ITS region has highest probability among all the markers used in case of correct identification for broader group of fungi. ITS markers are easier to amplify from small quantity of DNA as well as it has a high degree of variation even between closely related species [32]. Moreover, additional fungal studies have provided support for the ITS region as a suitable fungal barcode [33-35].

For DNA extraction, 1g of mycelia (fresh weight) was taken from 7 days old culture grown on PDA media. The genomic DNA was then isolated by using MaxMaxwell® 16 LEV Plant DNA Kit (AS1420, Promega, USA). The isolated DNA was then subjected to PCR technique to amplify specific region of the extracted DNA where universal primers, ITS4 (5'-TCCTCGCTTAT TGATATGC -3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') were used. In case of PCR amplification, 25 µl of a reaction mixture containing 2 µl genomic DNA, 2.5 µl 1X PCR buffer, 1.0 µl MgCl<sub>2</sub>, 1.5 µl dNTPs, 0.5 µl of each primer, 0.5 µl of Taq polymerase and 16.5 µl of deionized water were used [36]. Afterward, the PCR was programmed with an initialization step at 95 °C for 2 min, followed by 32 cycles of denaturation at 95 °C for 30 seconds, primer annealing at 48 °C for 30 seconds, and extension at 72 °C for 45 seconds and a final extension at 72 °C for 10 minutes. PCR products were separated by electrophoresis on 1.5% agar gel with 0.5% Ethidium bromide in 1x TAE buffer and visualized under UV light. After that the amplified DNA was purified, followed by sequencing in sequencing service laboratory, National Institute of Biotechnology (NIB), Bangladesh. The sequenced nucleotide data were analyzed through the BLAST program provided by the National Center for Biotechnology information (NCBI) (<http://blast.ncbi.nlm.nih.gov>) and finally, phylogenetic tree was constructed by using the data.

#### 2.6. *In vitro* screening of extracts presenting antifungal activity

Many studies revealed the antimicrobial properties of numerous plant species with increasing attention on herbal, medicinal, and aromatic plants for their antifungal activities [37]. For evaluating antimicrobial effect of plant extracts on the growth of isolated fungi, seven plant species were applied namely China rose, common fig, drumstick, guava, neem, ringworm shrub and scarlet gourd. For conducting the study, around 2 g cleaned fresh leaves were sterilized with 0.1% mercuric chloride followed by washing with distilled water. Thereafter, methanol (10 ml) was used as solvent. After that the leaves were grounded by mortar-pestle and subjected to centrifugation for 10 minutes (12000 rpm), which was then filtered through muslin cloth, followed by Whatman filter paper. After filtration, the supernatant was stored into refrigerator at 4 °C.

For evaluating antifungal activity of the leaf extracts, slightly modified poisoned food technique [38] was applied where 50 µg of plant extract were added at 20 ml of PDA medium. Then, medium-extract mixture was properly shaken and poured into petri plates. A fungal plug (6 mm diameter) was cut from 7 days old fungal culture and inoculated at the center of each Petri plate to investigate their inhibitory effectiveness on the growth of the fungal strain. The Petri dish inoculated with fungal disc alone served as control. Lastly, the Petri plates were incubated at 25±2 °C for 7 days in static

condition. Subsequently, the inhibition of the growth was calculated by Arora and Dwivedi method [39]. According to the method, and the inhibition percentage of mycelial growth=  $[(G_c - G_t)/G_c] \times 100$

Where,  $G_c$  = Mycelial growth in case of colony diameter in control set,

$G_t$  = Mycelial growth in terms of colony diameter in treatment set

### 3. Results

#### 3.1. Isolation of the pathogen

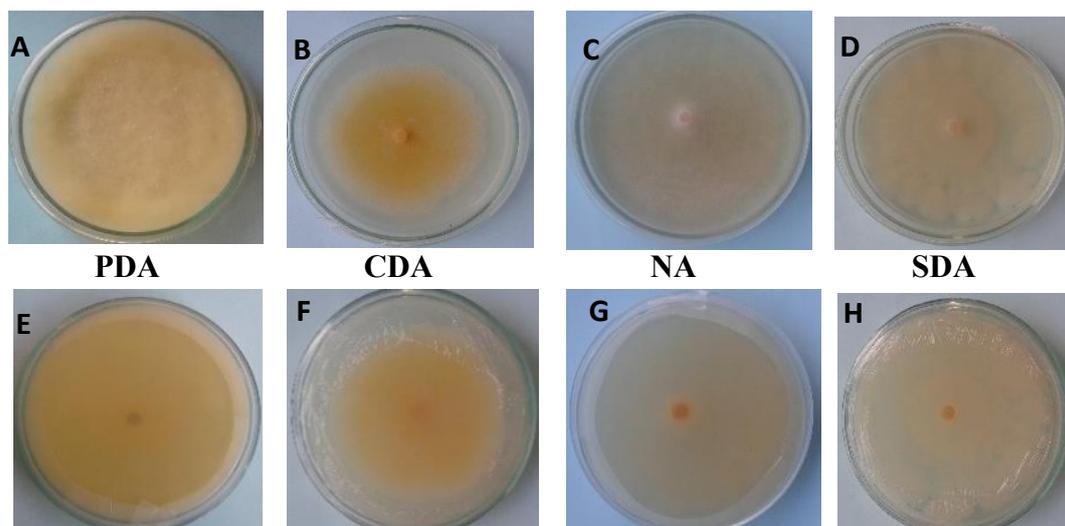
In our study, after sterilization collected sample was incubated on PDA medium that followed by single spore technique, and at the end of seven days, yellow colored fungal colonies were appeared. Through the procedure, the fungal pathogen was isolated and culture as single colony (images are not given).

#### 3.2. Morphological and microscopic identification

All four culture media showed variability in the growth of the isolated fungi to various degrees (table 1). The fungus showed maximum dry weight of 482 mg on PDA media after 7 days of incubation period. Moreover, differences in surface and reverse colouration of fungal colonies were distinct on the growth media (figure 2). The study of cotton blue staining revealed that sporangiophores bearing sporangia were hyaline, unbranched, apically dilated to form a clavate vesicle. The monosporous sporangia were brown in colour, ellipsoid to ovoid, 10–14  $\mu\text{m}$  in width and 11–20  $\mu\text{m}$  in length.

**Table 1** Colony morphology of the fungal strain on different media

Characteristics	Potato dextrose agar	Czapek agar	Dox	Nutrient agar	Sabouraud dextrose agar
Form	Filamentous	Filamentous		Filamentous	Filamentous
Margin	Filiform	Filiform		Filiform	Undulate
Elevation	Raised	Flat		Curled	Flat
Surface	Filiform	Filiform		Filiform	Filiform
Opacity	Non opaque	Non opaque		Non opaque	Non opaque
Front colour	Yellow	Yellow		Off white	Light yellow
Back colour	Flaxen	Flaxen		Off white	Light yellow
Dry weight (mg)	482	153		376	202



**Figure 2** Effects of the fungal isolate on different media; aerial mycelial growth (A, B, C, D) and the vegetative mycelial growth (D, E, F, G)

### 3.3. Growth profiling of the fungal isolate

#### 3.3.1. Effect of temperatures on the growth of the fungal strain

The fungal growth of the strain was estimated in 15 °C, 20 °C, 25 °C, 30 °C and 35 °C where it achieved highest growth at 25 °C. On the other hand, the minimum growth was observed at 35 °C (figure 3A).

#### 3.3.2. Effect of pH on the growth of the fungal strain

From the study, it was established that pH 5 was the most favorable pH for the mycelial growth whereas pH 7 provided least helpful environment to the fungal isolate for growth (figure 3B).

#### 3.3.3. Effect of carbohydrates on the growth of the fungal strain

In the study, the highest dry weight of the fungi was nearly 419 mg in case of dextrose containing culture media while the lowest was 347.33 mg having lactose in PDA (figure 3C).

#### 3.3.4. Effect of sugar concentrations on the growth of the fungal strain

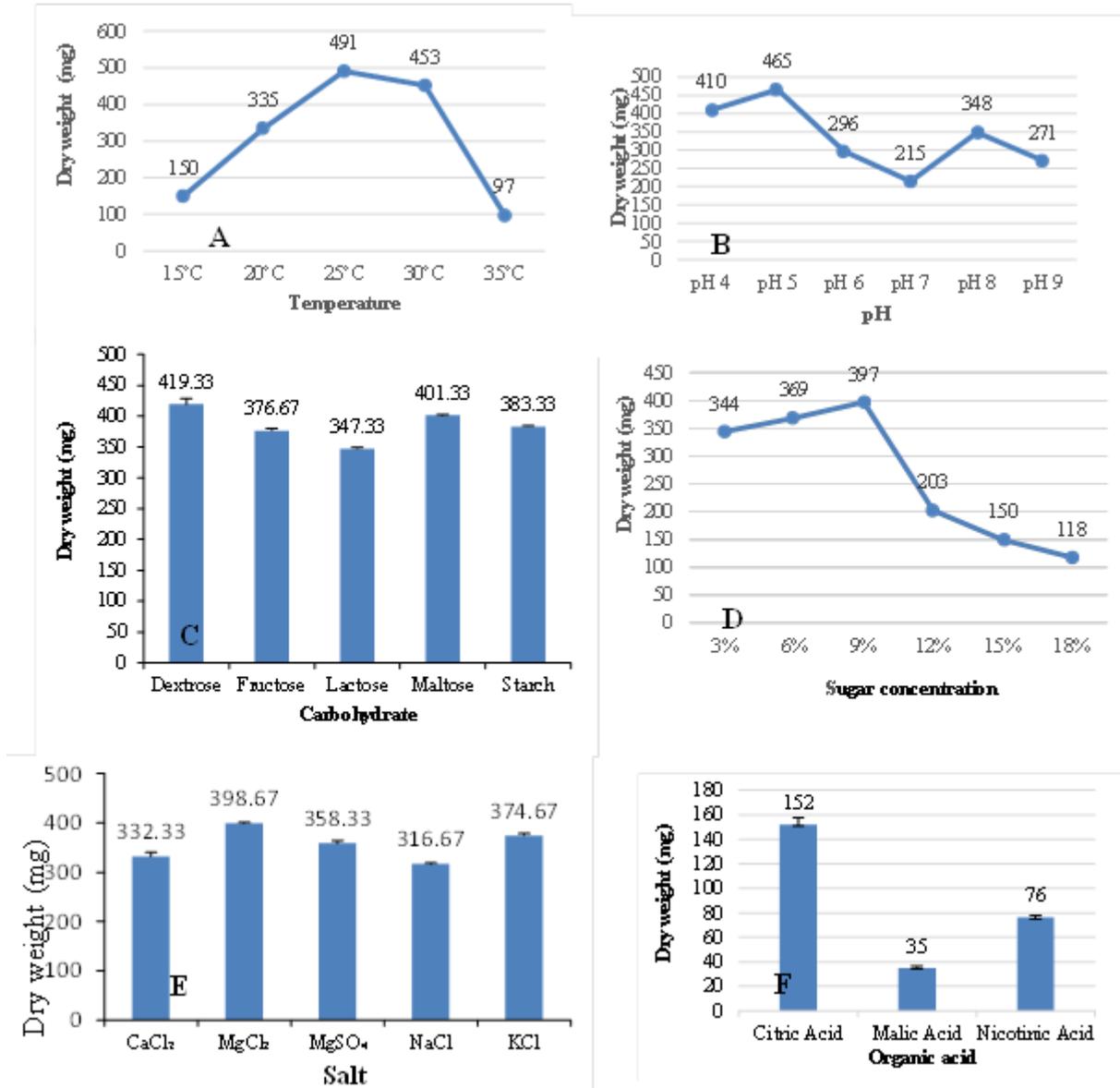
In the experiment, the fungal isolate was subjected to several sugar concentrations to evaluate the effect of sugar concentrations on the growth of the fungus. The mycelial dry weight for 3%, 6%, 9%, 12%, 15% and 18% sugar concentrations were 344 mg, 369 mg, 397 mg, 203 mg, 150 mg, and 118 mg, respectively (figure 3D).

#### 3.3.5. Effect of salts on the growth of the fungal strain

In case of the concerned fungal isolate, the highest growth was noted down for the salt, MgCl<sub>2</sub> whereas the NaCl showed lowest mycelial growth (figure 3E).

#### 3.3.6. Effect of organic acids on the growth of the fungal strain

The fungi grew well in citric acid at 1% concentration in comparison to other acids tested. On the other hand, the growth decreased most in malic acid and the dry weight was 35 mg (figure 3F).

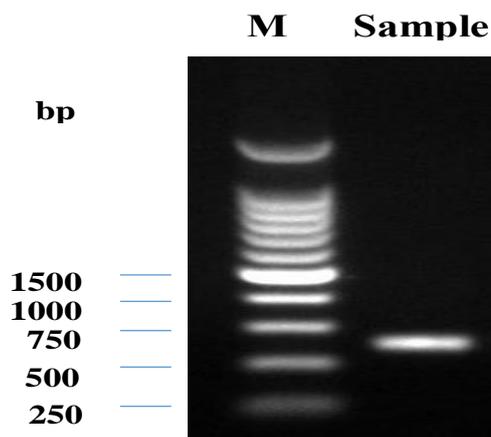


**Figure 3** The effects of different parameters on growth pattern of isolated fungi; (A) temperatures, (B) pH, (C) carbohydrates, (D) sugar (E) salts and (F) organic acids.

### 3.4. Molecular characterization

#### 3.4.1. PCR amplification

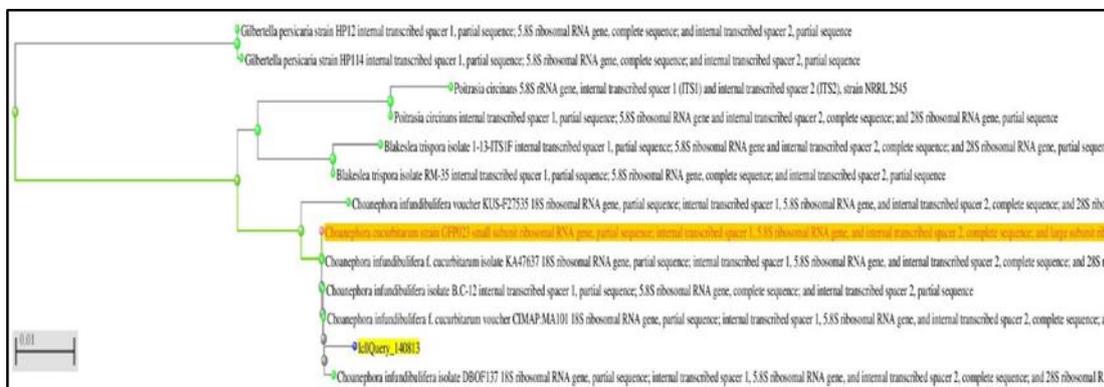
Upon extraction of the genomic DNA using DNA isolation kit, the extracted DNA was run on 1% agarose gel and visualized in gel documentation system. The 'ITS' region of ribosomal DNA was amplified through PCR technique with ITS4 and ITS5 primers and it produced approximately 650 bp bright band where 1kb DNA ladder was used as marker (figure 4). The sequenced 'ITS' region was then subjected to BlastN search in GenBank (<https://www.ncbi.nlm.nih.gov>) for proper identification of the isolate.



**Figure 4** PCR amplification of ITS region yielded ~650 bp product, 1kb DNA marker (M) is used for size determination

### 3.4.2. Sequencing and phylogenetic tree construction

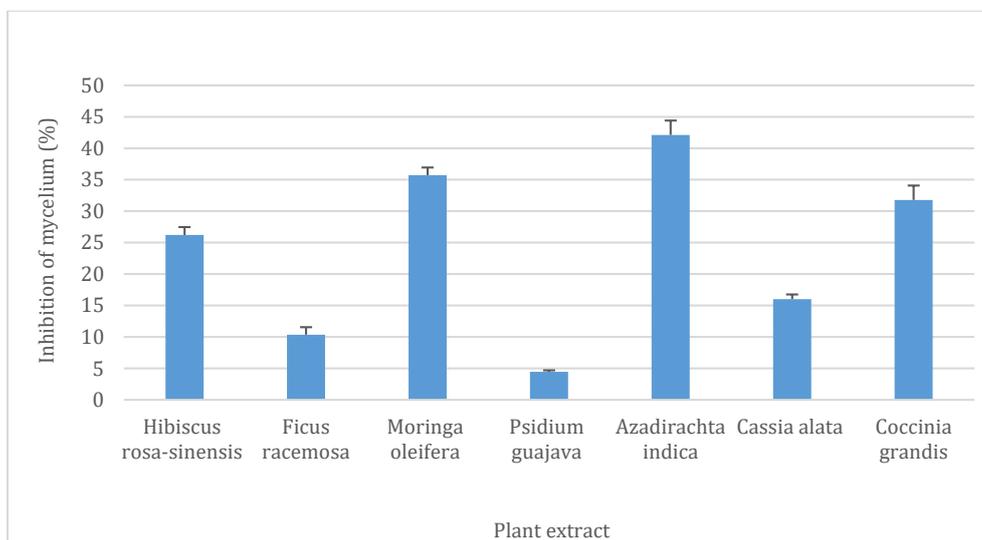
The 18S of rDNA sequence of the fungal strain revealed 99% similarity with the original sequence of *Choanephora cucurbitarum* (data not shown). To draw phylogenetic relationship, the sequences of different *Choanephora* spp. were downloaded in FASTA format from the GenBank and the data was then aligned to construct phylogenetic tree (figure 5).



**Figure 5** Phylogenetic relationship of the isolated fungal strain

### 3.5. In vitro screening of extracts presenting antifungal activity

In the experiment, seven different types of plants extracts were used (figure 6). Among them, *Azadirachta indica* (42.12%) and *Moringa oleifera* (35.71%) had highest inhibitory effects on the isolated fungus. Moreover, *Coccinia grandis* (31.78%), *Hibiscus rosa-sinensis* (26.21%) and *Cassia alata* (16.01%) had prominent negative effects on the growth of the fungus, but the *Psidium guajava* (4.44%) and *Ficus racemosa* (10.35%) had very low antifungal effects on the fungal isolate in comparison to other plant extracts.



**Figure 6** The effect of plant extracts on the inhibition of mycelial growth of the isolated fungi

#### 4. Discussion

Agriculture is the backbone and synonymous to the food security of a country [40]. In particular, it is the contributor of income and employment generation in Bangladesh [41]. Therefore, most of the farmers are marginally occupying subsistence farming and crop production raises rural income and creates jobs for poor people [42]. According to many researchers, crop losses are admittedly major threat to the wellbeing of rural families, to the economy of traders and governments, and to food security worldwide [43-45]. At the same time, crop losses due to pests and diseases for major food and cash crops were estimated between 20% and 40% at country and regional levels in different continents [46-47].

Bitter gourd is suffering from various diseases of which the fungal disease, *Choanephora* fruit rot caused by *Choanephora cucurbitarum* has become one of considerable constraints in bitter gourd growing area resulting in poor yields and reduced quality. Kacharek et al., [13] also reported that *Choanephora cucurbitarum* is a plant pathogenic fungus causing fruit rots, flower rot and leaf blights on a variety of plants.

Pathogen was isolated from the symptomatic infected fruit part on potato dextrose agar (PDA) under the temperature of  $25 \pm 2$  °C which was similar with the study of Saroj et al., [48]. In our experiment, PDA media was proved as most effective culture media to support mycelial growth of the fungal isolate. Several researchers [49] also stated PDA to be the best media for mycelial growth which was consistent with our findings. In our study, 25 °C was the suitable temperature for the fungi, while the optimum pH was 5. Kuo et al., [50] and Abdel-Motaal et al., [51] stated 25 °C-30 °C temperature as optimum for mycelial growth of *Choanephora cucurbitarum* which supported our observation. From the previous studies, it was established - acid/alkaline requirement for growth of fungi is quite broad, ranging from pH 3.0 to more than pH 8.0, with optimum around pH 5.0 if nutrient requirements are satisfied [52]. It was observed from our experiment that the maximum growth was achieved in dextrose and in case of 9% sugar concentration, respectively. In general, increasing in concentration of dextrose sugar up to 2.0% gave least response for mycelial growth, whereas further increase in concentration of dextrose sugar did not prove feasible. Our findings were in conformity with other experts [48, 53] who studied the carbon requirement on *Pleurotus ostreatus* and *P. florida* and showed that dextrose was the best carbon source. The addition of organic acid and salt significantly decreased the mycelial growth of our fungal isolate. The effect of organic acids on the fungal growth has been investigated by several authors [54-56]. Similarly, several studies have dealt with the use of different salt compounds to control various post-harvest diseases of *Citrus* and other crops [57-59].

The molecular identification of fungus was performed by sequencing of the ITS regions [60]. A nucleotide BLAST search of the publicly available fungal database showed that the 622 bp sequence of the ITS-rDNA region exactly matched with the *Choanephora cucurbitarum* with 99% similarity. Phylogenetic analysis was done with comparative analysis of different ITS-rDNA regions of NCBI published databases.

Plant extracts are now superior choice to control different plant pathogens that have been reported by several researches [61-63]. From our experiment, it was found that methanolic plant extract of neem, drumstick, scarlet gourd etc showed highest inhibitory effects against the fungal strain. The findings are consistent with the findings of Singh et al., [64] who reported that fungicidal and bactericidal properties of extracts from neem leaves either *in vitro* or *in vivo* trials to the presence of several antimicrobial active ingredients in leaves of neem tree such as desactylimbin, quercetin and sitosterol. Meanwhile, according to Ayanbimpe et al., [65], saprophytic fungi were also found to be inhibited by the extract of *Moringa oleifera* leaves.

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## 5. Conclusion

In the present investigation, morphological characteristics of the fungal isolate unfolded a great extent of differences when the fungus was grown in varied conditions of culture medium and environmental factors. The inequality in the nutrition revealed about their optimum requirements to achieve the highest growth as well as provided information about the unfavorable factors in case of their mycelial growth. Along with these studies, the molecular technique sequencing was supplemented for proper identification of the fungal isolate which disclosed the identity of the fungal isolate as *Choanephora cucurbitarum* which is the causal agent of the disease, *Choanephora* fruit rot in bitter gourd that responsible for significant economic loss of the bitter gourd producing farmers. At the same time, some plant extracts displayed commendable inhibitory effects against the pathogenic fungal strain which may assist to prevent the exaggerated disease of bitter gourd in near future.

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## Compliance with ethical standards

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### *Disclosure of conflict of interest*

The authors declare that there is no conflict of interests regarding the publication of this paper.

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