

# Black Stem of Sunflower: An Emerging Fungal Disease Caused by *Fusarium solani* in Bangladesh

Md. Maniruzzaman Sikder, Popy Akter, Md. Sabbir Ahmmed, Beauty Akter, Nusrat Binte Alam, Sayma Sajuti and Md. Nuhu Alam

Department of Botany, Faculty of Biological Sciences, Jahangirnagar University, Savar, Dhaka-1342, Bangladesh

## ABSTRACT

**Background and Objective:** Sunflower is one of the world's most important oil seed crops. However, these crops are prone to biotic factors, which reduce the yield of the crops. The present experiment was designed to identify and assess the growth characteristics of the fungal pathogens associated with the black stem disease of sunflowers and its control measures in Bangladesh. **Materials and Methods:** Both morphological features and molecular characterization based on the rDNA ITS region of the fungus were employed to identify the fungal pathogen. Six distinct solid fungal culture media, five separate temperature ranges and five different pH levels of medium were used to assess the mycelial growth behavior of the isolated fungus. Selected biocontrol agents and chemical fungicides were employed against the fungus under *in vitro* conditions. **Results:** The BLASTn search on the NCBI database revealed that the fungal pathogen (accession number: PP573854.1) showed above 99% homology with previously deposited sequences of *Fusarium solani*. Koch's postulate test was conducted to prove the pathogenicity. The fungal culture media, Potato Sucrose Agar and Carrot Agar exhibited the maximum mycelial growth. The vegetative growth of the fungus was most favored at 30°C temperature. Besides, slightly acidic conditions were mostly preferred by the *F. solani*. Among the fungal antagonists, *Trichoderma erinaceum* inhibited the maximum (81%) of the mycelial growth of the targeted fungus, followed by 73% for *Trichoderma harzianum*. Fungicides Amistar Top 325 SC and copper blue 50 WG showed above 70% growth inhibition against the vegetative growth under *in vitro* conditions. **Conclusion:** Biocontrol agents and synthetic fungicides will be promising options to control the black stem diseases of sunflowers. Black stem disease on sunflowers caused by *Fusarium solani* is a new record in Bangladesh.

## KEYWORDS

Black rot, potato sucrose agar, acidic pH, *Trichoderma erinaceum*, Amistar Top

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## INTRODUCTION

The sunflower (*Helianthus annuum* L.) is a flowering plant from the Asteraceae family. It is native to the North American Region<sup>1</sup>. It is one of the main vegetable oil sources globally, ranking after rapeseed, palm and soybean<sup>2</sup>. It is an important oilseed crop, contains a good percentage of oil (48-53), protein (14-19), crude fiber (16-27), ash (2-3), soluble sugar (7-9) and hull (21-27). Its short duration and



photo-insensitivity suit it well for cultivation in the rainy season<sup>3</sup>. The sunflower is cultivated globally as an oil seed crop which yields one of the world's most important sources of cholesterol-free, unsaturated edible oil with high vitamin E content. For this reason, the popularity of this oil is increasing day by day worldwide.

Various biotic factors are responsible for the economic losses of sunflowers. Several fungal pathogens cause major diseases of the crop. *Fusarium* spp. was previously considered a minor pathogen in sunflowers<sup>4</sup>. However, *Fusarium* spp., *F. oxysporum* var. *orthoceras*; *F. sporotrichioides*, was found to be serious pathogens in sunflowers in different states of the USA, Argentina and Russia where damage up to 80% has been reported by Gulya *et al.*<sup>5</sup> and Leslie and Summerell<sup>6</sup>. Besides, a survey was undertaken in the U.S. (Minnesota, North Dakota and South Dakota) and Argentina in 2010 to determine stalk rot pathogens of sunflowers; aggressiveness of the eight species of *Fusarium*, in which *F. equiseti* and *F. sporotrichioides* were the most aggressive species, followed by *F. acuminatum*<sup>7,8</sup>. In Bangladesh, Sikder *et al.*<sup>9</sup> reported sunflower stem rot diseases and are responsible for lowering seed production in Bangladesh. However, researchers in our country have not conducted a comprehensive study on sunflower black stem disease. The present study was conducted to detect the black stem-causing fungal pathogen of sunflower plants via morphological and molecular approaches; to assess the effect of fungal culture media and environmental factors on the vegetative growth of the isolated fungus; to evaluate the impact of bio-control agents and fungicides against the targeted fungus.

## MATERIALS AND METHODS

**Sample collection and isolation of fungal pathogen:** At the beginning of the research work, several farmer's fields located in Mankigonj District, Bangladesh were visited to visually observe disease incidence during July and August, of 2021. After the rainy seasons, blackish symptoms appeared on the stem above the base of the sunflower plants. At least 10 diseased samples were collected in sterilized zipper bags from the selected fields. Those samples were washed in running tap water several times and dried on sterile Petri dishes. Next, samples were chopped into small pieces, followed by surface sterilization using 5% sodium hypochlorite for 5 min. Then, samples were washed several times with sterile water to remove disinfectants. Afterward, the samples were air-dried, placed on potato dextrose agar (PDA) medium and incubated at room temperature (25±2°C) for 7 days. The hyphal tip method was used to isolate purified fungal pathogens.

**Identification of pathogen:** Firstly, morphological features such as colony characters, pigmentation, hyphal septation and branching pattern, conidia and conidiophore structure, chlamydospores formation pattern, etc. were investigated. Besides, the genomic DNA of the isolated fungus was extracted according to the procedure described by Sultana *et al.*<sup>10</sup>. Polymerase Chain Reaction (PCR) was performed to amplify the internal transcribed spacer (ITS) region of the fungus using specific primer sets ITS5 (5-GGAAGTAAAAGTCGTAACAAGG-3) and ITS4 (5-TCCTCCGCTTATTGATATGC-3) as the method described by White *et al.*<sup>11</sup>. To amplify the fungal ITS sections, a PCR reaction was conducted in a 25 µL volume having 1 µL of a DNA template, 12.5 µL of a reaction mixture containing dNTPs, Buffer, MgCl<sub>2</sub>, Taq Polymerase (Origin: Promega, USA), 1 µL each of forward and reverse primers (20 pMol) and 9.5 µL H<sub>2</sub>O. The PCR cycles consist of an initial denaturation at 95°C for 3 min, 35X (95°C for 30 sec, 48°C for 30 sec and 72°C for 50 sec), termination at 72°C for 5 min and overnight hold at 4°C<sup>9,12</sup>. The PCR product was run on agarose gel, then purified using PCR Clean-Up System (Promega, USA) and sent to FIRST BASE Laboratories (Sdn Bhd, Kuala Lumpur, Malaysia) for sequencing. The neighbor-joining tree was generated using the MEGA 11 version 11.0.13 software.

**Koch's postulates for pathogenicity:** To fulfill Koch's postulates, an *in vivo* experiment was carried out in the experimental site of the Botanical Garden, Department of Botany, Jahangirnagar University, Savar, Dhaka-1342, Bangladesh. The healthy seedlings were grown in potting media. After 45 days, the stems

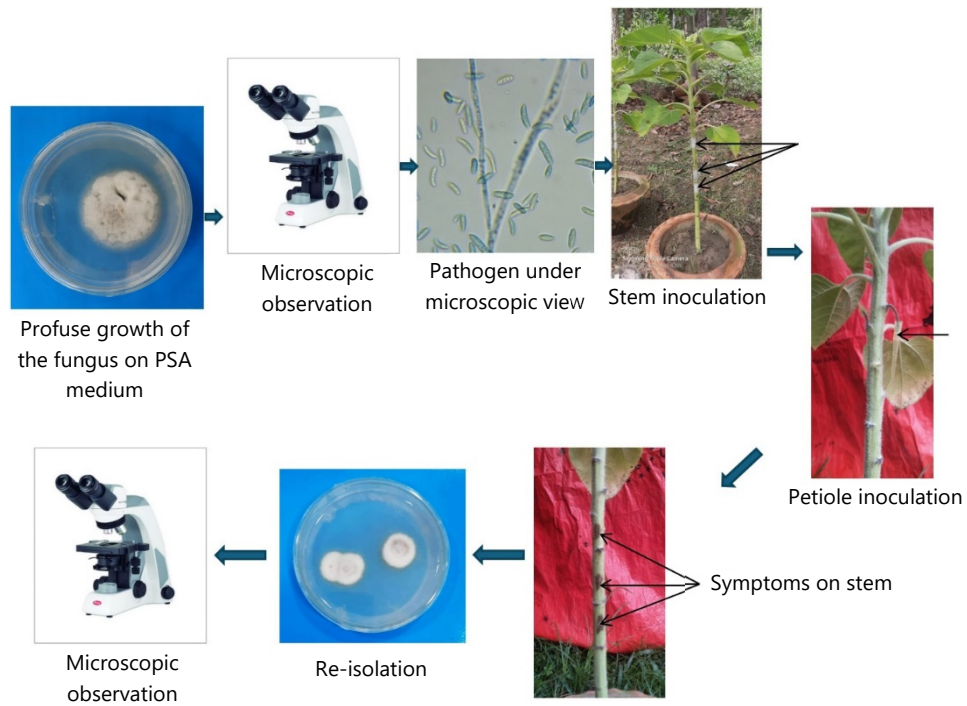


Fig. 1: Pathogenicity test of the isolated fungus

of five plants were injured using a sterile blade and the mycelial plug was inoculated on the stem and carefully wrapped with parafilm (Fig. 1). The leaf blades of five sunflower plants were removed using a sterile blade and the petiole was placed in a fungal inoculum-loaded eppendorf tube (200  $\mu$ L). The plants treated without inoculum served as a control. Those plants were kept under controlled environmental conditions for 2 weeks. After initiating the disease symptom, re-isolation of the fungal pathogen was done to prove the pathogenic nature of a particular fungus.

**Evaluation of the effects of culture media, temperature and pH on fungal growth:** Culture media have different nutrient compositions that influence the metabolism of the fungus. The impact of six unlike fungal culture media viz., potato sucrose agar (PSA), carrot agar (CA), sabouraud dextrose agar (SDA), yeast extract agar (YEA), honey peptone agar (HPA) and hansen's agar (HA) on the mycelial growth of the fungus were assessed as mentioned by Sikder *et al.*<sup>13</sup>.

Temperature is known to stimulate fungal growth and proliferation. The role of five different temperature conditions viz., 15, 20, 25, 30 and 35°C on the mycelial growth of the identified fungus was assessed as described by Ahmmed *et al.*<sup>14</sup>.

Substrate pH also determines the fungus's growth and development. The effect of five different pH levels (5.0, 6.0, 7.0, 8.0 and 9.0) on the fungus's radial growth characteristics was determined as mentioned by Sultana *et al.*<sup>15</sup>.

**Efficacy of antagonistic fungi and fungicides against the tested fungus:** For an eco-friendly disease management system, antagonistic microorganisms are the best tools. The isolated fungus was tested against three *Trichoderma* spp., viz., *T. harzianum* (accession number: MH368147.1), *T. erinaceum* (lab collection) and *T. asperellum* (accession number: MH368120.1)<sup>16</sup>. A 2 mm diameter agar disc containing tested fungus of 7 day old cultures and 7 day culture of *Trichoderma* isolates were placed an equal distance in the Petri plate containing PDA. Three replications were maintained during the study. Only the centrally placed tested fungal disc was inoculated on the control plate. The inoculated plates were sealed

with parafilm and incubated at  $25\pm 2^{\circ}\text{C}$  temperatures for 7 days. The radial mycelial growth was measured after 7 days of incubations. The radial mycelial growth of the experimented plates was calculated and compared with the control plate.

The growth inhibition percentage of the tested fungus was determined using the following formula<sup>13</sup>:

$$I = \frac{C - T}{T} \times 100$$

Where:

I = Mycelium growth inhibition (%)

C = Vegetative growth in the control plate

T = Vegetative growth in the treatment plate

The effect of three different fungicides, namely Mycoguard 80 WP (mancozeb+metalaxyl), copper blue 50 WG (copper oxychloride) and Amistar Top 325 SC (azoxystrobin+difenoconazole) on the mycelial growth of isolated fungus using the food poison technique<sup>17</sup>. The requisite amounts of fungicides were mixed into a PSA medium with a concentration of Mycoguard at 1000, 1500 and 2000 ppm; copper blue at 2000, 2500 and 3000 ppm and Amistar Top at 1000, 1500 and 2000 ppm. Fungicides were mixed with the media after autoclaving. Six replicates of PSA plates were used for every dose of the respective fungicide. The PSA plate that received no fungicide served as control. Each Petri plate was inoculated with the mycelial disc of the test fungal pathogen and was taken from the 7-day-old colony on PSA. The inoculated plates were incubated at  $25\pm 2^{\circ}\text{C}$ . The colony diameter was recorded after 7 days post-inoculation (dpi). The mycelial growth inhibition percentage was calculated using the previously described formula.

**Statistical analysis:** Data was analyzed using the statistical software SPSS-16 at a 5% level of significance. The phylogenetic tree was generated using MEGA 11 version 11.0.13 software.

## RESULTS

**Identification and pathogenicity of the fungus:** The black discoloration appeared on the developing sunflower stem and extended to the petiole (Fig. 2a). The fungal colony was circular depending on culture

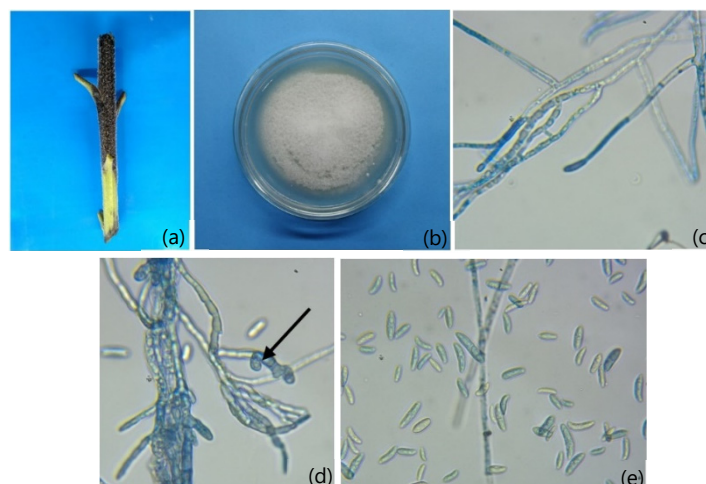


Fig. 2(a-e): Morphological characteristics of (a) *Fusarium solani* causing black stem disease on sunflower, (b) Symptoms of *Fusarium solani* on the stem and (c-e) Pure culture of the fungus on PDA medium

Microscopic view of mycelium, chlamydospore and microconidia (100 10x), respectively

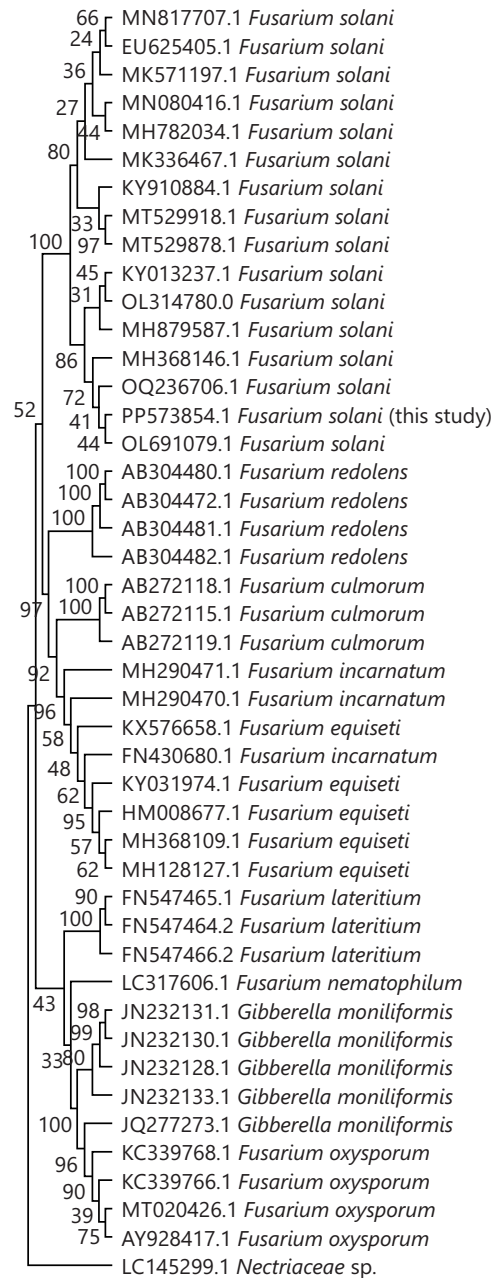


Fig. 3: Neighbor-joining tree derived from analysis of ITS sequence dataset of the studied organism with bootstrap value (bootstrap replication = 1000)

NCBI accession numbers are mentioned before the genus names and our organism is marked with "This study"

media, with abundant white-colored, cottony/wooly appearance and elevated conidial masses with smooth margins (Fig. 2b). The mycelia were septate, hyaline and abundantly branched under microscopy (Fig. 2c). Chlamydospore formation was found (Fig. 2d). The microconidia evolved on separate, hyaline conidiophores that were well-developed. Microconidia were hyaline, oval-ellipsoidal shaped and aseptate (Fig. 2e), while macroconidia and acervuli were not found.

In the BLAST search, the ITS sequence of our fungal pathogen (PP573854.1) showed above 99% similarity with *F. solani*, which suggested the taxonomic position of the studied fungus. In the neighbor-joining tree, there were six distinct clusters of the different species of *Fusarium*, where the currently studied fungal pathogen *F. solani* (PP573854.1) was grouped into a separate cluster with *F. solani* clade with a bootstrap value of 100 (Fig. 3).

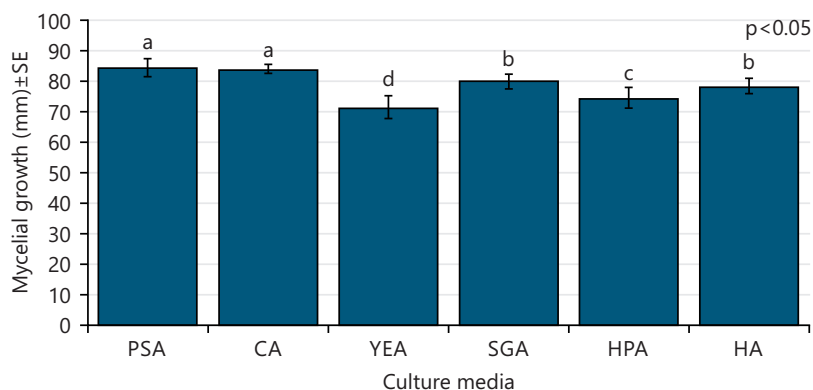


Fig. 4: Effect of culture media on the mycelial growth of *F. solani* at 7 dpi

Value represents the Mean ± Standard Error (SE) of six replications. The significant difference is presented as the alphabet on the top of data points and the same letter does not differ significantly (DMRT), \* $p < 0.05$ . PSA: Potato sucrose agar, CA: Carrot agar, YEA: Yeast extract agar, SGA: Sabouraud dextrose agar, HPA: Honey peptone agar and HA: Hansen's agar

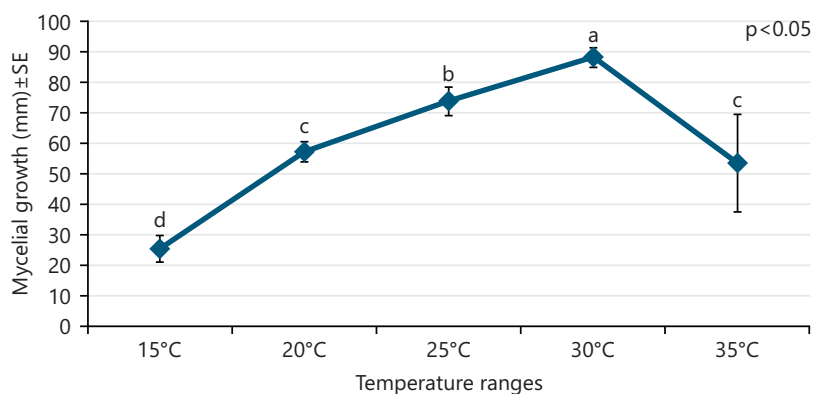


Fig. 5: Effect of temperature on the mycelial growth of *F. solani* at 7 dpi

Significant difference is presented as the alphabet on the top of data points and the same letter does not differ significantly (DMRT), \* $p < 0.05$  and the value represents the Mean ± Standard Error (SE) of six replications

Typical black stem symptoms were observed on the stem; however, no symptoms were not found on the petiole. The pathogenic nature of *F. solani* with black stem disease of sunflower was confirmed through the pathogenicity test.

**Fungal biology of the studied organism:** The media components are important criteria for fungal culture and study and important physiological parameters that lead to maximum sporulation in fungi. The effect of six different solid media viz., potato sucrose agar (PSA), carrot agar (CA), yeast extract agar (YEA), sabouraud dextrose agar (SGA), honey peptone agar (HPA) and hansen's agar (HA) on mycelial growth of *F. solani* was evaluated (Fig. 4). The results showed that there is a significant effect of culture media on the mycelial growth of *F. solani* ( $p < 0.05$ ) and the highest mycelial growth (84.25 mm) was obtained on PSA medium which was statistically similar to CA medium (83.78 mm), followed by SGA (79.78 mm) and HA (78.22 mm) media and the lowest mycelial growth (71.22 mm) on YEA medium (Fig. 4). The colony was whitish, cottony, elevated and smooth margin on PSA, whitish, cottony, emerged and smooth margin on CA, whitish, woolly, emerged and smooth margin on YEA, SGA, HPA, off-white, cottony, emerged and smooth margin on HA medium.

The impact of different temperature conditions assessed on the vegetative growth of *F. solani* on PSA media under *in vitro* conditions (Fig. 5). The colony appearance was changed with the increasing temperatures, such as light yellow at 15°C, whitish grey at 20°C, whitish on the top side while brown at



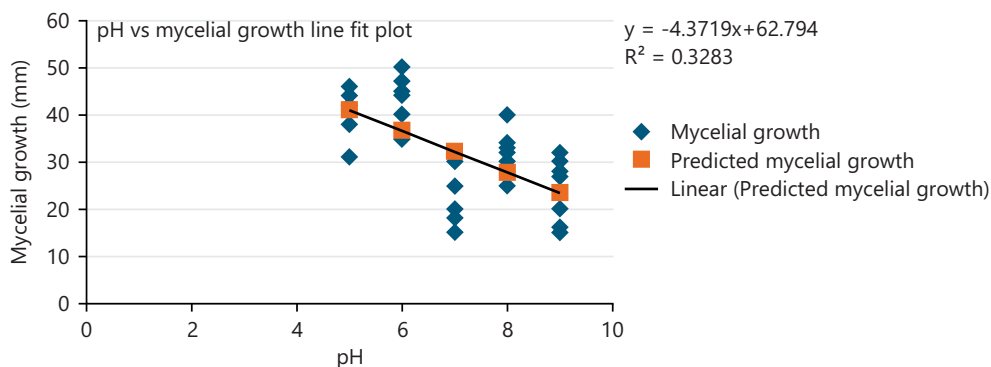


Fig. 6: Effect of pH on the vegetative growth of *F. solani* at 7 dpi  
Value represents the mycelial growth of six replications

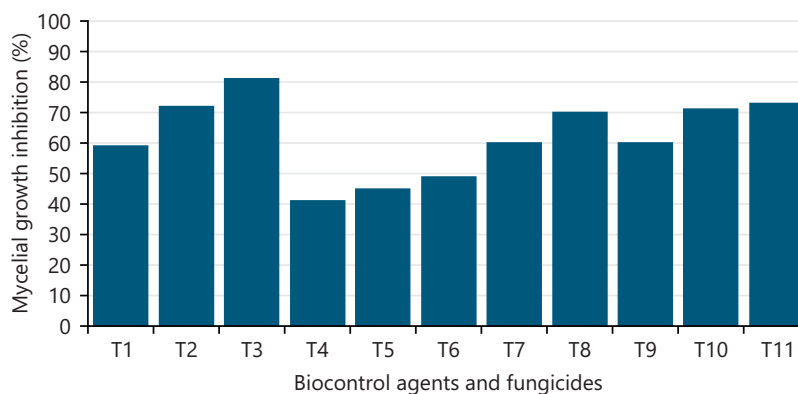


Fig. 7: Effect of biocontrol agents and chemical fungicides against the growth of *F. solani*

Value represents a percentage of six replications. Here, T1: *T. asperellum*, T2: *T. harzianum*, T3: *T. erinaceum*, T4: Mycoguard 1000 ppm, T5: Mycoguard 1500 ppm, T6: Mycoguard 2000 ppm, T7: Copper blue 2000 ppm, T8: Copper blue 2500 ppm, T9: Copper blue 3000 ppm, T10: Amistar top 1000 ppm, T11: Amistar Top 1500 ppm and T12: Amistar top 2000 ppm

the bottom side at 30°C and whitish at 35°C. Data revealed a significant difference in response due to temperature conditions ( $p < 0.05$ ). There was an upward trend of vegetative growth of *F. solani* up to 30 and afterward started to decline. The maximum mycelial growth (88.22 mm) was obtained at 30°C, followed by 25°C (73.92 mm) and 20°C (57.12 mm) while the lowest growth (25.44 mm) was found at the extreme lowest temperature (15°C).

The experimental plates were incubated at five different pH levels viz., 5, 6, 7, 8 and 9 to find suitable pH conditions for the fungus. Data revealed that *F. solani* showed the maximum mycelial growth at acidic to neutral conditions (Fig. 6). The growth rate was comparatively slower at pH 8-9. However, pH conditions explained only 32% variation in the mycelial growth of the studied fungus.

**Role of antagonistic agents and fungicides to the fungus:** Bio-control agents (*Trichoderma harzianum*, *T. erinaceum* and *T. asperellum*) showed promising results against the fungus. Among the three antagonists, *T. erinaceum* showed the maximum mycelial growth inhibition (81%) of *F. solani* followed by *T. harzianum* (73%) and *T. asperellum* (59%) (Fig. 7). The present study was also aimed to screen out the most effective chemical fungicides viz., Mycoguard 80 WG (mancozeb+metalaxyl), copper blue 50 WG (copper oxychloride) and Amistar Top 325 SC (azoxystrobin+difenoconazole) in low concentration against *F. solani* which cause black stem disease of sunflower. The result revealed that all three fungicides are effective against the fungus at moderately high concentrations in the current study. The growth inhibition percentage was highest (73%) at 2000 ppm concentration of Amistar Top, followed by 71 and 60% due

to 1500 and 1000 ppm, respectively. Coper blue performed best (70%) at 3000 ppm concentration, followed by 68% for 2500 ppm and 60% for 2000 ppm concentration. Almost 50% growth inhibition was obtained at the 2000 ppm concentration of Mycoguard, followed by 1500 and 1000 ppm (Fig. 7).

## DISCUSSION

Based on morphological characteristics, it was identified as *Fusarium* sp. Identifying the fungus at species rank without molecular characterization was difficult. Molecular techniques were used to determine the species rank. A BLASTN search showed that the fungus could be *Fusarium solani*. After generating the phylogenetic tree, we confirmed the fungus's taxonomic position. Sultana *et al.*<sup>18</sup> also used similar techniques to identify the *Fusarium* at species rank.

In the current experiment, *F. solani* showed the highest mycelial growth in PSA and CA medium, which is supported by Mallik *et al.*<sup>19</sup>, who noted the 75.8 and 74.0 mm mycelial growth of *F. solani* in PSA and CA media, respectively. Osono and Takeda<sup>20</sup> stated that reduced glucose content in fungal culture media inhibits the expansion of quickly developing species and promotes sporulation, which facilitate the identification of various fungal species. Observable morphological characteristics remain the basic foundation of fungal systematics. Thus, the phenotypes of fungi are used to identify and recognize them. Likewise, Lazarotto *et al.*<sup>21</sup> also recorded the utmost vegetative growth and sporulation of *Fusarium chlamydosporum* in the PSA medium. Sultana *et al.*<sup>18</sup> found the highest vegetative growth of *Fusarium fujikuroi* species complex on honey peptone agar, PSA and PDA media. Another bioassay revealed that the PDA and CA culture media were appropriate for *Fusarium equiseti* causing wilt disease of *Centella asiatica*<sup>22</sup>. Therefore, PSA, CA and PDA media are most suitable for the vegetative growth of *F. solani*.

Current study results revealed that the 30°C temperature was mainly appropriate for the vegetative growth of *F. solani*. Previous bioassays also showed that the temperature range between 25 to 30°C was most suitable for growth and sporulation of *F. solani*, *F. tricinctum* and *F. oxysporum*<sup>23-25</sup>. Akter *et al.*<sup>22</sup> reported the maximum mycelial growth of *F. equiseti* at 25°C temperature. Another study concluded that a temperature of 22°C or above was reported as an optimum for *F. oxysporum*<sup>26</sup>. Hence, investigation suggests that 30°C is most suitable for the proliferation of the mycelium of *F. solani*.

In the present study, *F. solani* grew better at slightly acidic conditions, which conforms with the findings of Gordon *et al.*<sup>27</sup> reported that the frequency of root infection of the strawberry cultivar Albion due to *Fusarium oxysporum* f. sp. *fragariae*, was significantly higher for plants grown in acidified soil (near pH 5) than soil near neutrality. Likewise, Groenewald *et al.*<sup>28</sup> noted the most suitable growth of different isolates of *Fusarium oxysporum* f. sp. *cubense* at a pH level of 5-6. Another lab bioassay<sup>29</sup> showed the optimum growth and survival of *Fusarium oxysporum* f. sp. *elaedis* was found between 5.0 and 7.0. In other lab studies, neutral to slightly alkaline conditions were most suitable for the vegetative growth of *Fusarium oxysporum*<sup>30-32</sup>. Current results suggested that an increased pH level of the substrate could limit the growth of the pathogen at an economic level; however, more studies are needed to confirm this.

The most promising results were obtained when biocontrol agents were used to restrict the vegetative growth of the fungus. Around 80 and 70% of mycelial growth inhibition of the targeted fungus were found by *T. erinaceum* and *T. harzianum*, respectively. Previous results suggest that *T. erinaceum* bio-priming modulated the defense mechanism in plants along with the accumulation of higher antioxidative enzyme activities after challenging through *F. oxysporum*<sup>33</sup>. Besides, Herath *et al.*<sup>34</sup> assessed the antagonistic activity of *T. erinaceum* against plant pathogenic fungi and found promising results. Biocontrol agent *T. erinaceum* was found to be an effective fungus in controlling several soil-borne plant pathogens (i.e., *Rhizoctonia solani*, *Sclerotium rolfsii* and *Sclerotium oryzae*) both under *in vitro* and *in vivo* conditions<sup>35</sup>. Rojo *et al.*<sup>36</sup> reported on the biological control of *F. solani* causing peanut brown root rot disease by



*Trichoderma* sp. and found that *T. harzianum* is an effective biological control agent. Lian *et al.*<sup>37</sup> reported that *T. harzianum* conidia has been proven to restrict the growth of *F. oxysporum*, reduce disease progression and enhance crop productivity. Several reports suggested that *T. harzianum* was an effective antagonist against soil-borne fungal pathogens<sup>38-40</sup>. Furthermore, a previous study indicated that *T. asperellum* was shown to be an effective biocontrol agent against *Fusarium oxysporum* f. sp. *niveum*<sup>41</sup>. Hence, biocontrol agents could be used to restrict the vegetative growth of *F. solani*.

Both chemical fungicides, viz., Amistar Top 325 SC (azoxystrobin+difenoconazole) and copper blue (copper oxychloride), showed a significant reduction in the vegetative growth of the fungus. In Egypt, Al-Kahal *et al.*<sup>42</sup> reported that copper oxychloride was the most effective against *F. solani* causing root rot disease of faba bean. Mallik *et al.*<sup>19</sup> reported that Amistar Top 325 SC showed more than 50% mycelial growth restriction of *F. solani* by 750 ppm concentration. Masoud *et al.*<sup>43</sup> studied that the fungicide Azoxystrobin provides the best results against targeted fungi. The highest level of inhibition was found using the fungicide Azoxystrobin (Amistar 25% SC). Data revealed that Amistar Top 325 SC inhibited (88.9%) mycelia growth of *F. solani*. Hence, current study results suggest that Amistar Top and copper blue are effective fungicides that could be applied to control the mycelial growth of *F. solani*.

## CONCLUSION

The black stem of sunflowers caused by *F. solani* is emerging as a significant fungal disease, which is becoming a serious threat to the cultivation of sunflowers in Bangladesh. This disease has the potential to significantly reduce sunflower yields, hampering regional attempts to improve oilseed production. Correct identification of the disease-causing organism was done using both molecular and morphological approaches. Pathogens preferred specific environmental conditions such as temperature of 30°C and pH 5-6. Integrated disease management practices, including the use of resistant varieties, early sowing, change of soil pH and judicious application of fungicides, incorporation of bio-pesticides are essential for controlling the pathogen. To provide long-lasting solutions and viability of sunflower crops in Bangladesh, more study and observation are required.

## SIGNIFICANCE STATEMENT

Sunflower is one of the most edible oil-yielding crops. Farmers face heavy losses due to fungal infestations. Correctly identifying the fungus is an essential step in formulating management practices. In this study, both morphological and molecular techniques were applied to identify the fungal pathogen. The pathogenic nature of the identified fungus was also determined. Results revealed that 30°C temperature was an ideal condition for the mycelial growth of the fungus. Lab bioassay revealed that biocontrol agents, viz., *Trichoderma erinaceum* and *Trichoderma harzianum* and fungicides Amistar Top and copper blue, were effective against the isolated fungus. The results of this present study will help to formulate the effective management of black stem diseases and enhance the quality of seed production of sunflower plants.

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