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Alternaria leaf spot of broccoli caused by *Alternaria alternata* in Bangladesh

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Abstract: This study aimed to isolate and characterise the pathogen associated with Alternaria leaf spot on broccoli and to evaluate the inhibitory effects of fungicides against it. We isolated and identified the fungal pathogen as *Alternaria* sp. using morphological and cultural methods. Based on the aligned sequences of the internal transcribed spacer (ITS) and molecular phylogenetic analysis by the neighbour joining method, the isolates (Ab1 and Ab2) were confirmed as *Alternaria alternata*. The conidia of the isolates were dark brown, cylindrical, obclavate to muriform. The conidiophores were olivaceous brown, septate, and branched. The conidial morphology of the isolates ranged from 52.4–92.4 × 10–20 µm with 2–6 transverse and 0–3 longitudinal septa. Both isolates yielded positive results in the pathogenicity test on broccoli leaves by developing brown and circular spots with concentric rings on the leaves surrounded by yellow halos. The culture studies revealed that the maximum growth of the pathogen was obtained at 30 °C and pH 6.0. Tilt 250 WC showed the highest potential in suppressing the mycelial growth of the *A. alternata in vitro* at a concentration as low as 50 µg/mL. The results from this study contributed to the positive identification of the pathogen and characterised *A. alternata* as a destructive pathogen of broccoli which may be successfully controlled by the fungicide Tilt.

Keywords: fungicide; conidia; internal transcribed spacer; phylogenetic analysis; Tilt

Broccoli (*Brassica oleracea* var. *italica*) is one of the most popular vegetables in Bangladesh because it naturally contains bioactive phytochemicals such as glucosinolates, phenolic compounds, vitamin C and mineral nutrients (Kaur et al. 2007). Climatic constraints and a number of diseases are the limiting factors for broccoli production in Bangladesh. Leaf spot caused by *Alternaria* spp. is an important and severe disease of brassicas worldwide (Meah et al. 2002; Kirk et al. 2008). The first symptom of the disease appears with minute yellow specks on mature leaves and stems. Isolation and molecular characterisation is an essential tool for diagnosis of *Alternaria* spp. which will eventually help in selecting efficient control measures against the pathogen.

In Bangladesh, leaf spot has become a destructive disease of broccoli which may lead to economic losses of this winter vegetable. However, there is no report found on the isolation, morphological characterisation and molecular identification of *A. alternata* from broccoli in Bangladesh thus far. Very little information is available regarding its management using fungicides. Therefore, the objectives

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of this study were to isolate and identify the leaf spot pathogen using morphological and molecular methods and cultures, and to evaluate the efficacy of a commercial fungicide in controlling the pathogen.

MATERIAL AND METHODS

Isolation and morphological characterisation of the leaf spot pathogen. Broccoli leaves having brown and circular spots with concentric rings surrounded by yellow halo symptoms were collected from a farmer's field of the Gazipur district in Bangladesh. To isolate the causal organism of the leaf spot disease, the infected portions of the leaves from the advancing symptom areas were cut into small pieces and sterilised in a 0.1% NaOCl solution for one minute. The pieces were washed repeatedly three times in sterile distilled water and then transferred onto a sterilised potato dextrose agar (PDA) medium (Hase and Nasreen 2017) and also into test tube slants under aseptic conditions and then incubated at room temperature (27 ± 1 °C) for seven days and observed periodically for fungal growth. Colonies which developed from the bits were identified by microscopic observation by taking note of the mycelial and conidial characteristics. To purify the pathogen, the conidial suspension was prepared in sterilised distilled water from the 10-day-old PDA culture. Two microlitres of the conidial suspension were spread uniformly on 2% water agar plates. These plates were incubated at 27 ± 1 °C for 12 h and then the germinating conidium was cut and transferred to a PDA plate. The growing hyphal tip was again transferred to fresh PDA plates. The obtained pure isolates were properly labelled and preserved in a refrigerator at –20 °C. Two isolates namely Ab1 and Ab2 were used for the subsequent studies. The conidia of the Ab1 and Ab2 isolates were taken from the pure culture and mounted on a clear glass slide over which a cover slip was placed. The shape and size of the conidia were observed and photomicrographs were taken.

Confirmation of the pathogenicity of the isolates. The pathogenicity of two isolates (Ab1 and Ab2) was tested on the broccoli variety 'BARI Broccoli-1' in this study. Ten broccoli seeds were sown and three pots were used for each isolate. Four leaf stage seedlings were sprayed with the conidial suspension. For spraying, the inocula of both fungal isolates were adjusted to 1×10^5 conidia/mL. The non-inoculated plants were sprayed with sterilised water. The inoculated plants were covered with sterilised transparent polyethylene bags and kept under natural light conditions at 25–30 °C. Those bags were removed after 48 h and the symptoms of the infection were checked periodically until the characteristic symptoms appeared. Re-isolation of the pathogen was undertaken using the same procedure used in the pathogen isolation and was found to have identical morphological characteristics of the tested organisms.

DNA extraction and quantification of the isolated pathogen. The genomic DNA was extracted from the Ab1 and Ab2 isolates following a modified CTAB (hexadecyltrimethylammonium bromide) method. Briefly, the isolates were grown on a PDA medium for 10 days at 25 °C and the mycelia were harvested by scraping the media. The harvested mycelia were then ground in a mortar with a pestle adding 600 µL of extraction buffer (33 mM CTAB; 0.1 M Tris-HCl, pH 8.0; 7.8 mM EDTA; 0.7 M NaCl). The suspension was collected in an Eppendorf tube and incubated at 65 °C for 30 min in a block heater with occasional shaking. An equal volume of phenol : chloroform : isoamyl alcohol (25 : 24 : 1) was added to separate the DNA containing solution from the cell debris and then the DNA was precipitated using cold isopropanol. Then a DNA pellet was obtained by centrifuge and washed two times with 70% ethanol, dried under a vacuum and re-suspended in nuclease free water adding 10 µg/mL of RNAase A and incubated at 37 °C for 30 minutes. The extracted DNA was quantified using a fluorometer (Qubit 4; Thermo Fisher Scientific, USA).

Amplification of conserved region of the genome of the isolates. The conserved internal transcribed spacer region (ITS1, 5.8S and ITS4) of the genome of the fungi was amplified by polymerase chain reaction (PCR) using a gradient thermal cycler (Verti 96 well thermal cycler; Applied Biosystems, USA). The isolated DNA was used as template for a total volume of the 50 µL PCR mixture. The universal primers ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') were used for the amplification (Tarbell 2008). A slightly modified thermal cycle from Tarbell 2008 was used for the amplification. The thermal cycle was an initial denaturation for 2 min at 94 °C followed by 35 cycles of denaturation for 45 s at 94 °C, annealing for 30 s at 60 °C,

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extension for 2 min at 72 °C and a final extension step of 10 min at 72 °C. To determine the amplification, 8 µL of the PCR amplicons were mixed with 2 µL of 6X loading dye (0.25% bromophenol blue and 40% sucrose in double-distilled water) and loaded in a 1% agarose gel containing ethidium bromide as a DNA staining agent with a molecular weight standard marker (1Kb plus ladder marker; Thermo Fisher Scientific, USA). Then electrophoresis was performed in a 1X Tris-Borate-EDTA buffer for 40 min at 80 V. The amplicons were visualised with UV light in an illuminator.

PCR product purification and DNA sequencing. The amplicons were purified by using a commercial Gene JET PCR Purification Kit (Thermo Fisher Scientific, USA) following the manufacturer's protocol. The purified PCR products of the Ab1 and Ab2 isolates were sent to Macrogen Inc., South Korea for sequencing. After completion of the sequence, the assembled sequence of each isolate was subjected to a BLAST analysis in the National Center for Biotechnology Information (NCBI) database and the phylogenetic tree of the isolates was constructed using MEGA 7 software (version 7.0). The sequences were submitted to the NCBI GenBank to obtain the accession numbers.

Effect of temperature and pH on the mycelial growth of the isolates. Mycelial disks of each isolate (5 mm in diameter) were placed on PDA plates and were incubated in a growth chamber at six different temperatures (15 °C, 20 °C, 25 °C, 30 °C, 35 °C and 40 °C).

The PDA media were adjusted to pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0, respectively. The inoculated plates were incubated at 27 ± 1 °C with each treatment and replicated five times. After five days, the colony diameter was measured.

***In vitro* bioassay of the fungicides on the radial growth of *Alternaria* sp.** Five fungicides namely Ridomil MZ 68 WP (metalaxyl 40 g/kg + mancozeb 640 g/kg), Rovral 50 WP (iprodione 500 g/kg), Autostin 50 WGD (carbendazim 500 g/kg), Dithane M-45 (mancozeb 750 g/kg) and Tilt 250 EC (propiconazole 250 g/L) were tested *in vitro* to evaluate their effect on the colony growth of both isolates following the Poison food technique on a PDA medium (Dhingra and Sinclair 1985). The medium was poured into conical flasks, 100 mL per 250 mL flask. Before solidification, a requisite quantity of the individual fungicide except Tilt 250 EC was added to the PDA to have concentrations of 100 ppm, 150 ppm, 200 ppm, 250 ppm and 300 ppm. However, Tilt 250 EC was evaluated at the concentrations of 5 ppm, 15 ppm, 25 ppm, 50 ppm and 75 ppm. Approximately 15 mL of melted PDA mixed with fungicides was poured into each 90 mm Petri dish. After solidification, the plates were inoculated by placing 5 mm discs of the 7-day-old culture. Each treatment was replicated five times. The plates were incubated at 27 ± 1 °C data on the colony diameter was recorded after seven days of inoculation when the growth of the fungi of the control plates completely covered the plate. The diameter of the colonies on the PDA with and without the fungicide was measured from the upper side of the Petri dishes. The inhibition percentage was calculated based on the growth of the pathogen on the treated and control PDA plates following the formula as stated by Sundar et al. (1995):

$$\text{Inhibition \%} = (A - B)/A \times 100$$

where: A – mycelial growth of the pathogen in absence of the fungicides; B – mycelial growth of pathogen in presence of the fungicides.

Statistical analysis. The Statistix version 10 and Microsoft Office Excel 2010 program packages were used for the statistical analysis. The experimental design was completely randomised, consisting of five replications for each treatment.

RESULTS

Isolation and morphological identification of the pathogen. All of the examined isolates produced light green to greenish dark coloured mycelia. The colonies were surrounded by white young tip hyphae at the initial growth stage that later turned greenish black in colour. The conidia of both isolates were almost the same in shape. The conidia of the isolates were dark, cylindrical, obclavate and muriform. The conidiophores were olivaceous, septate, and branched. However, there was a slight difference in the sizes of the conidia of the two isolates. The dimension of the conidia of the isolates ranged from 56.6–92.4 × 10–20 µm, 52.4–85.7 × 15–20 µm (Figure 1) with 2–6 transverse and 0–3 longitudinal septa.

Pathogenicity test of the *Alternaria* isolates. Spots with concentric rings on the inoculated leaves surrounded by yellow halos were observed

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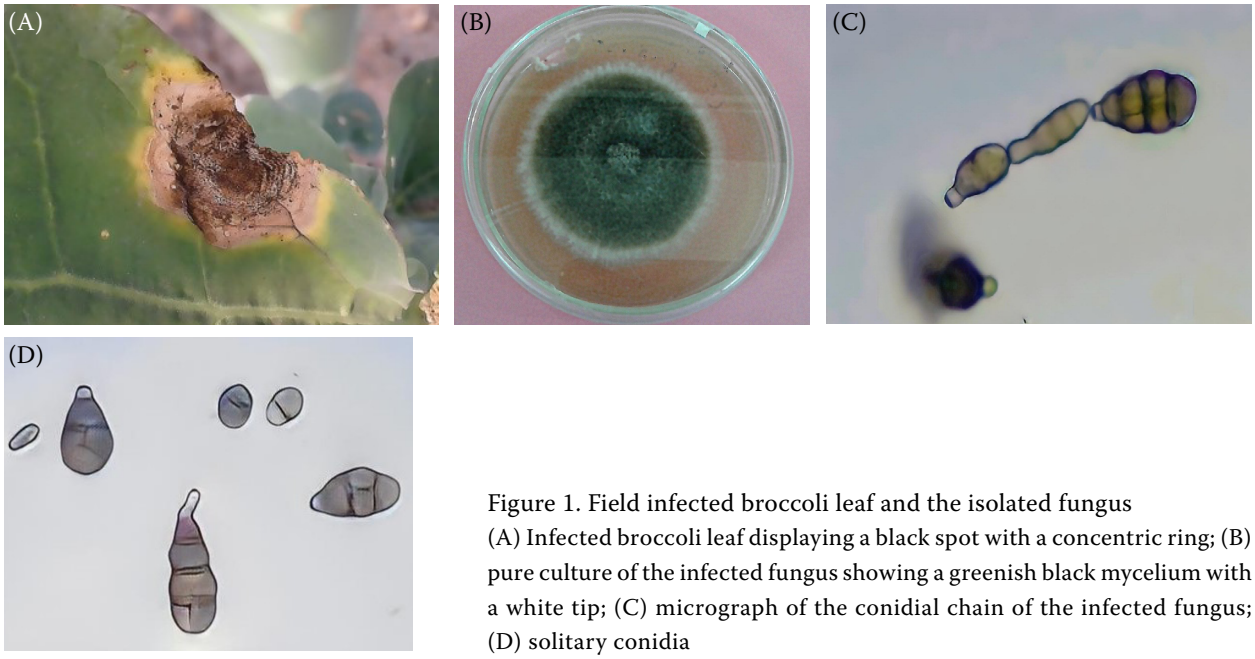

Figure 1. Field infected broccoli leaf and the isolated fungus
(A) Infected broccoli leaf displaying a black spot with a concentric ring; (B) pure culture of the infected fungus showing a greenish black mycelium with a white tip; (C) micrograph of the conidial chain of the infected fungus; (D) solitary conidia

at 15–25 days after inoculation (Figure 2B). The inoculated fungus was re-isolated from these artificially inoculated leaves. The plants, which were inoculated with water only, remained healthy and symptomless.

Molecular identification of the pathogen. Amplification of the ITS region of Ab1 and Ab2 gave a distinct band (460 bp) in the gel electrophoresis. The sequencing results revealed that the two isolates showed 99% sequence homology with *Alternaria alternata* in the BLAST search as well as in the neighbour joining method tree (Figure 3) based on their ITS regions. The ITS sequences of Ab1 and Ab2 were submitted to the NCBI GenBank with accession numbers MT102712 and MT102716, respectively.

Effect of temperature on the mycelial growth of the *Alternaria* sp. isolates. The isolates exhibited varying responses to the different treatments. The maximum mycelial growth was recorded at 30 °C for isolate Ab1 (49.00 mm) and Ab2 (48.80 mm) which was significantly superior to the other treatments. The second highest growth was observed at 25 °C for isolate Ab1 (41.40 mm) and Ab2 (40.80 mm) which was followed by 20 °C. Poor mycelial growth was found at 35 °C for both the isolates. The least growth was shown to be at 15 °C for isolate Ab1 (14.40 mm) and Ab2 (14.50 mm), however, no growth was recorded at 40 °C (Table 1).

Effect of the different pH level on the mycelial growth of the *Alternaria* sp. isolates. The significantly highest mean mycelial growth of isolate

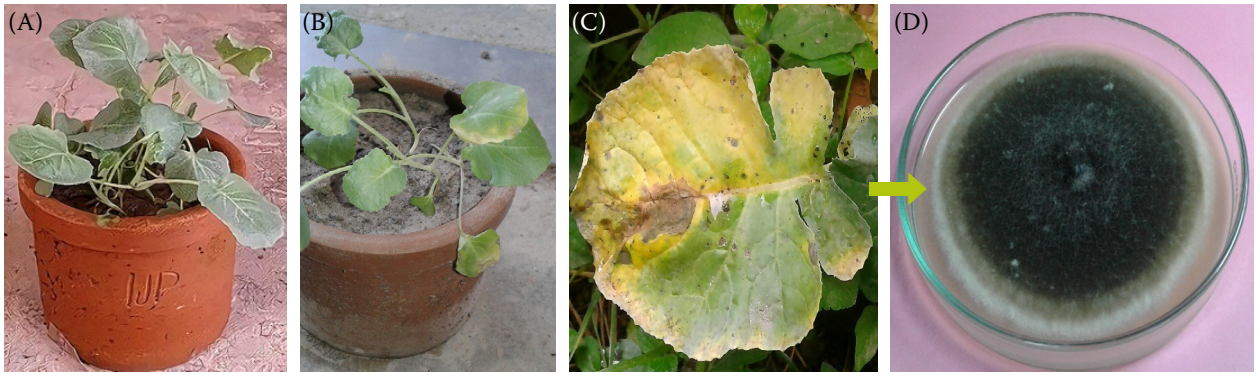

Figure 2. Pathogenicity test of *Alternaria* sp. against BARI Broccoli-1
(A) Control plant; (B and C) diseased plant after being treated with *Alternaria* sp.; (D) culture of *Alternaria* sp. re-isolated from the artificially infected broccoli leaves

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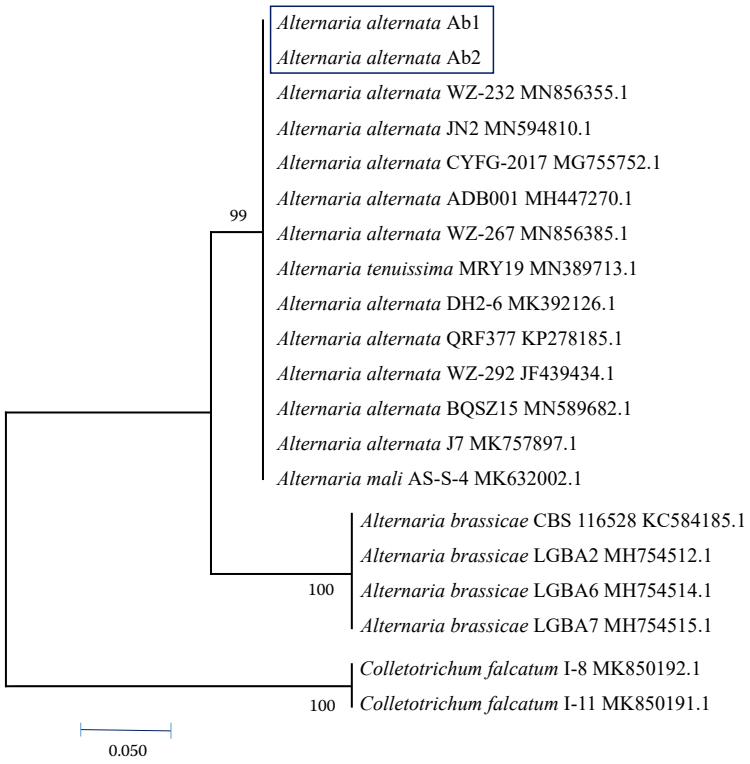

Figure 3. Neighbour-joining method tree based on the internal transcribed spacer sequences from the present isolates and other reference strains of *Alternaria brassicae*

Number on the nodes represent the bootstrap value

Ab1 (55.00 mm) and Ab2 (55.60 mm) were recorded at pH 6.0. The colony diameter of isolate Ab1 was 49.00 mm at pH 5.5, which was followed by pH 5.0 (43.60 mm), pH 6.5 (41.80 mm), pH 7.0 (34.80 mm), pH 7.5 (34.40 mm), pH 8.0 (28.60 mm). The colony diameter of isolate Ab2 was 49.60 mm at pH 5.5, followed by pH 5.0 (42.25 mm), pH 6.5 (41.00 mm), pH 7.0 (36.60 mm), pH 7.5 (34.60 mm), pH 8.0 (29.80 mm) (Table 2).

Efficiency of the fungicides on suppressing the mycelial growth of the *Alternaria* sp. isolates. All five fungicides showed a significant inhibitory effect on the radial growth of the *A. alternata* isolates on the PDA plates at 100 ppm, 150 ppm, 200 ppm, 250 ppm and 300 ppm concentrations. The rate of inhibition varied with the fungicides and their concentration. Tilt 250 EC completely inhibited the growth of *Alternaria* sp. isolates at all the tested concentrations. With the increasing concentrations of the other fungicides, the percent of inhibition of the fungus also increased and the maximum inhibition was obtained at the highest concentration tested. In case of isolate Ab1, Rovral

Table 1. Mycelia growth of the *Alternaria* sp. isolates at different temperatures

Temperature (°C)	Colony diameter (mm)	
	Ab1	Ab2
15	14.40 ± 0.46^{e}	14.50 ± 0.56^{e}
20	30.80 ± 0.47^{c}	30.80 ± 0.60^{c}
25	41.40 ± 0.45^{b}	40.80 ± 0.59^{b}
30	49.00 ± 0.43^{a}	48.80 ± 0.50^{a}
35	21.60 ± 0.46^{d}	21.60 ± 0.61^{d}
40	0.00 ± 0^{f}	0.00 ± 0^{f}
CV (%)	2.74	3.40

Values are the mean ± SE. Different letters indicate significant differences at a 5% significance level

Table 2. Mycelia growth of the *Alternaria* sp. isolates at the different pH levels

pH range	Colony diameter (mm)	
	Ab1	Ab2
5.0	43.60 ± 1.10^{c}	42.25 ± 0.55^{c}
5.5	49.00 ± 0.96^{b}	49.00 ± 0.53^{b}
6.0	55.00 ± 0.95^{a}	55.60 ± 0.51^{a}
6.5	41.80 ± 1.08^{c}	41.00 ± 0.64^{d}
7.0	34.80 ± 1.12^{d}	36.60 ± 0.49^{e}
7.5	34.40 ± 1.10^{d}	34.60 ± 0.51^{e}
8.0	28.60 ± 0.81^{e}	29.80 ± 0.53^{f}
CV (%)	4.25	1.99

Values are the mean ± SE. Different letters indicate significant differences at a 5% significance level

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50 WP suppressed 86.31% and 94.14% of mycelial growth of the fungus at 100 ppm and 150 ppm, respectively. The mycelial growth was completely arrested at the 200 ppm, 250 ppm and 300 ppm concentrations of Rovral 50 WP. Dithane M-45 and Ridomil Gold MZ 68 WG provided moderate results, but poorer to Rovral 50 WP. Dithane M-45 reduced 34.27%, 38.66%, 45.61%, 53.32% and 60.43% of the colony diameter at 100 ppm, 150 ppm, 200 ppm, 250 ppm and 300 ppm concentrations, respectively. The reduction in the colony diameter were 31.22%, 36.24%, 42.20%, 49.69% and 55.41% under the treatment with Ridomil Gold MZ 68 WG at the 100 ppm, 150 ppm, 200 ppm, 250 ppm and 300 ppm concentration, respectively. Autostin 50 WGD showed less effectiveness against the mycelial growth inhibition compared to all the other fungicides. The inhibition percentages of the fungal growth were 20.10%, 26.24%, 31.43%, 37.29% and 45.23% at the 100 ppm, 150 ppm, 200 ppm, 250 ppm and 300 ppm concentrations of Autostin 50 WGD, respectively. Rovral 50 WP showed a better result against the Ab2 isolate than the Ab1 isolate. At 200 ppm, 250 ppm and 300 ppm concentrations, the fungal growth totally ceased. This fungicide was capable of reducing 88.12% and 94.40% of the mycelial growth of the Ab2 isolate at the 100 ppm and 150 ppm concentrations, respectively. Dithane M-45 showed 36.61%, 42.23%, 48.55%, 54.20% and 61.43% inhibition of the mycelial growth at the 100 ppm, 150 ppm, 200 ppm, 250 ppm and 300 ppm concentrations, respectively. Ridomil Gold MZ 68 WG provided 31.87%, 35.20%, 39.44%, 46.23% and 52.29% inhibition at the 100 ppm, 150 ppm, 200 ppm, 250 ppm and 300 ppm concentrations, respectively. The inhibition percentages of the mycelial growth with Autostin 50 WGD were 22.18%, 29.12%, 35.22%, 43.32% and 49.80% at the 100 ppm, 150 ppm, 200 ppm, 250 ppm and 300 ppm concentrations, respectively (Figures 4 and 5).

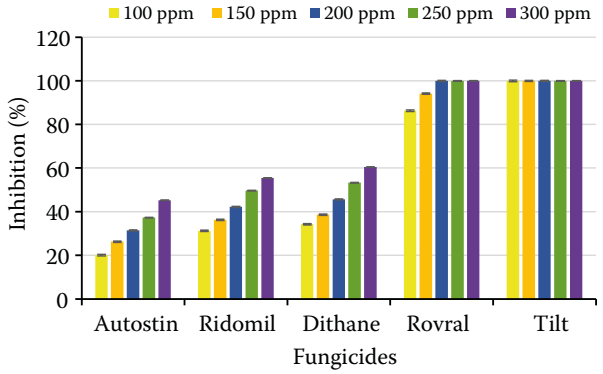

Figure 4. Inhibition percentage of the *Alternaria* sp. isolate Ab1 on the potato dextrose agar amended with different fungicides at different concentrations
Bars are the mean ± SE

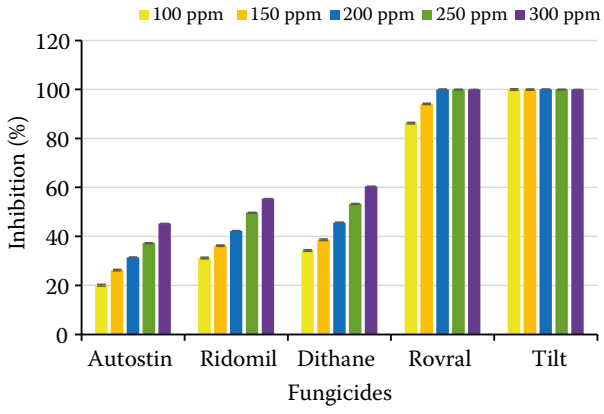

Figure 5. Inhibition percentage of the *Alternaria* sp. isolate Ab2 on the potato dextrose agar amended with different fungicides at different concentrations
Bars are the mean ± SE

As Tilt 250 EC is a highly toxic fungicide, it was further tested against *A. alternata* at 5 ppm, 15 ppm, 20 ppm, 25 ppm, 50 ppm and 75 ppm concentrations. This fungicide fully inhibited the fungal growth at a 50-ppm concentration. It caused 72.23%, 78.20% and 87.66% fungal inhibition of isolate Ab1 at 5 ppm, 15 ppm and 25 ppm, respectively. Tilt 250 EC also caused the highest percent inhibition of the mycelial growth in the case of Ab2. It resulted in a 73.66%, 80.00% and 88.15% reduction in the mycelial growth at the 5 ppm, 15 ppm and 25 ppm concentrations, respectively (Table 3).

Table 3. Inhibition percentage of the radial colony growth of the *Alternaria* sp. isolates on the potato dextrose agar amended with Tilt 250 EC at different concentrations

Concentrations (ppm)	Inhibition % of radial growth over control	
	Ab1	Ab2
5	72.23 ± 0.70^{d}	73.66 ± 0.71^{d}
15	78.20 ± 0.68^{c}	80.00 ± 0.72^{c}
25	87.66 ± 0.75^{b}	88.15 ± 0.69^{b}
50	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
75	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
CV (%)	2.92	2.60

Values are the mean ± SE. Different letters indicate significant differences at a 5% significance level

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DISCUSSION

Brassica seedlings are often infected by seed-borne pathogens, especially by the serious pathogen *Alternaria* spp. in many countries (Kubota et al. 2006; Rahimaloo & Ghosta 2015). This study, for the first time, isolated and characterised the pathogen of Alternaria leaf spot in broccoli in Bangladesh as *A. alternata*. The presence of concentric rings and black spots surrounded by yellow halos on the infected leaves also supports that the pathogen is *Alternaria* sp. (Verma & Verma 2010). The microscopic observations revealed that mycelium was septate and branched. Conidia usually produced singly at the apex of the conidiophores, but sometimes in the short acropetal chain (Singh et al. 2014). The morphological characteristic of both isolates produced light green to greenish dark colour mycelia. The colonies were surrounded by white young tip hyphae at the initial stage of the growth that later turned greenish black in colour. After inoculation with the pathogen, the test plants began to present symptoms typical of *Alternaria* sp., which was identical to those observed in the field (Humpherson-Jones 1992; Howard et al. 1994; Stammler et al. 2014; Devappa & Thejakumar 2016; Verma et al. 2018).

The morphological characteristics may provide clues for the accurate identification of *Alternaria* sp. Numerous studies have already demonstrated that the importance of comparative sequence-based strategies for fungal species identification in addition to using morphological characteristics. Studies have confirmed the effectiveness of the nuclear ribosomal internal transcribed spacer region (ITS1 and ITS4) sequences located between the nuclear small and large subunit rRNA genes for species complex level identification (Mahmoud & Zaher 2015). The data presented here demonstrate that the PCR amplification of the ITS gene fragment using a specifically designed primer, followed by a sequencing and phylogenetic analysis identified both the isolates as *A. alternata*.

A detailed characterisation of *A. alternata* and its biology is necessary for determining appropriate management strategies. The present study indicates that the fungal growth was good at a temperature range of 20 °C to 30 °C and the maximum growth was recorded at 35 °C. Previous studies reported that the best growth of *A. alternata* and other *Alternaria* spp. were at a temperature of 25–30 °C and a pH level of 6–6.5 pH, which supports the findings of this study (Hubballi et al. 2010; Dinh 2015; Azad et al. 2016).

To manage diseases, the application of fungicides is the principal response for most crops in order to manage Alternaria leaf spot. We screened the commonly available fungicides. Our data indicate a fair to good efficiency of Ridomil MZ 68 WP (metalaxyl + mancozeb), Rovral 50 WP (iprodione), Autostin 50 WGD (carbendazim), Dithane M-45 (mancozeb) and excellent for Tilt 250 EC (propiconazole). Tilt 250 EC completely inhibited the growth of *Alternaria* sp. isolates at the recommended doses. The lowest inhibition was observed in Autostin 50 WGD. The results of this experiment are similar to those of Arunakumara (2006), who reported that propiconazole showed the maximum inhibition of the mycelial growth of *A. brassicae* (84.75%). Kakraliya et al. (2018) observed the effect of fungicides against *Alternaria* sp. and a maximum inhibition percent of the mycelia growth was observed in propiconazole (89.72%). Datta and Gopal (1999) found that Rovral 50 WP was very effective in controlling *A. brassicae.* Valvi et al. (2019) also tested mancozeb 75% WP (0.25%), propiconazole 25% EC (0.1%) and carbendazim 50% WP (0.1%) *in vitro* to control *A. brassicae* causing Alternaria leaf spot of cauliflowers and found 100%, 96.29% and 3.70% growth inhibition, respectively.

CONCLUSION

In summary, this study, for the first time, isolated and identified a destructive pathogen of leaf spot of broccoli as *A. alternata* using morphological, molecular, pathological and culture analyses. Among the fungicides tested, Tilt 250 EC displayed the highest suppression of the pathogen. The information reported in this article should be useful for understanding epidemiology of the disease and implementing an effective management strategy against broccoli leaf spot caused by *A. alternata*. Further studies should include Tilt in a field trial and compare its efficacy with non-treated check.

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