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First report on leaf spot disease of *Aloe vera* caused by *Alternaria alternata* (Fr.) Keissler in Bangladesh

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Abstract
An experiment was conducted to find out fungal pathogen associated with leaf samples of *Aloe vera* obtained from commercial fields of Northern part of Bangladesh. Leaf spot disease causing fungus-*Alternaria alternata* was identified through morphological characterization based on mycelium, conidia, colony features as well as molecular characterization using internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) of fungi. Molecular identification revealed that ITS sequences of our studied fungus (MH368103.1) was genetically similar to sequences of *Alternaria alternata* in NCBI database. Typical leaf spot was reproduced by artificial inoculations of the isolated fungus. The mycelial growth of this fungus was evaluated on different culture media i.e., potato dextrose agar (PDA), carrot agar (CA), potato sucrose agar (PSA), Richard agar (RA), Honey peptone agar (HPA), Honey agar (HA); temperature (15, 20, 25, 30, 35^{0}C) and light condition (complete light, complete dark, alternate light-dark). *A. alternata* showed the maximum mycelial growth on PDA at 25^{0}C temperature. Both complete dark and alternate light-dark condition were mostly favored for profuse vegetative growth of the fungus. Fungal antagonist-*Trichoderma reesei* showed very promising results, which inhibited the mycelial growth under *in vitro* condition. Systemic fungicide Tilt 250EC (500ppm) showed complete inhibition of the vegetative growth of the studied fungus. To the best of our knowledge, *Alternaria alternata* causing *Aloe vera* leaf spot disease in Bangladesh, a new record.

Keywords: *Aloe vera*, *Alternaria alternata*, control measures, molecular identification

1. Introduction

Aloe vera (L.) Burm.f. is one of the important medicinal plants, which has been cultivating in Northern part of Bangladesh (Chowdhury *et al*, 2018) [9]. It is used in medicine, cosmetics and as juice. The contamination with fungal pathogen in the plants is of public importance. Some fungal pathogens and non-pathogens produce mycotoxins that infected their hosts and substrates on which they grow. Mycotoxins are hazardous to human and animal health, which can cause cancer, hemorrhage, edema and immune deficiency (Makun *et al*, 2010) [16].7 *Aloe vera* is susceptible to various pathogenic microorganisms, which causes quality loss and reduce the production of economic part of the plant. Leaf spot of *Aloe* caused by the genus *Alternaria* have been reported in different parts of the world; namely-leaf spot of *A. barbadensis* by *Alternaria alternata* in India (Chavan and Korekar, 2011) [8], in Lousinia, USA (Silva and Singh, 2012) [24], in Pakistan (Bajwa *et al*, 2010) [24]. In Bangladesh, Shamsi and Shutrodhar (2013) [23] recorded *Alternaria pluriseptata* as associated fungal pathogen on *Aloe vera* leaf. Nasreen *et al*, (2013) [18] reported four fungi from *Aloe vera*, namely-*Cochliobolus lunatus*, *Aspergillus* sp., *Penicillium* sp., and *Fusarium* sp. Leaf spot disease of *Aloe vera* caused by *Nigrospora oryzae* has reported recently in Bangladesh (Begum *et al*, 2018) [18]. The present experiment was attempted to detect and find out the causal organism of leaf spot of *Aloe vera*; to characterize both morphological and molecular features; to study of fungal biology of the pathogen, to evaluate the efficacy of antagonistic fungi, and chemical fungicides against isolated fungus.

2. Materials and Methods

Diseased *Aloe vera* leaves with characteristic symptom were collected from northern district (Natore) of Bangladesh and brought into laboratory. Tissue planting method was used to isolate fungal pathogen; subculture was maintained on PDA medium; the pure culture was stored in refrigerator at -4°C. Isolated fungi from the infected tissues of *A. vera* leaf samples was identified on the basis of colony morphology and morphological characteristic of conidia. Detached leaf method used to test pathogenicity of the fungus.

For molecular characterization, fungus genomic DNA samples were extracted using DNA extraction Kit (Promega, USA). DNA concentration was measured using Nano Drop Spectrophotometer (ND2000, Thermo Scientific, USA). The primer ITS4 (5'-TCCTCCGCTTATT GATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAA CAAGG-3') were used for the PCR reaction (White *et al*, 1990) [31]. The PCR reaction was performed with 20 ng of genomic DNA as the template in a 25 µl reaction mixture by using a LA Taq (TAKARA BIO INC, Japan) as follows-activation of Taq polymerase at 94°C for 5 minute, 35 cycles of 94°C for 30 Sec, 55°C for 30 Sec, and 72°C for 5 minutes each were performed, finishing with a 10-minute step at 72°C. The Maxwell® 16 DNA Purification Kits were used to purify the amplification products (Promega, USA). The purified PCR products of approximately 570 bp, sequenced by using two primers in First BASE Laboratories SdnBhd (Kuala Lampur, Malaysia). After necessary trimming from both ends of the DNA sequence, submitted to NCBI and received accession number.

DNA sequences were checked with Chromas and a BLAST search with the ITS sequences was used to reveal the closest matching taxa in family-Pleosporaceae, multiple sequence alignments done using MEGA 6. Data converted from fasta to MEGA format with Clustal W. The models of evolution were determined under the Akaike information criterion (AIC). The models selected were Tamura-3 parameter for analysis. Maximum likelihood (ML) analysis done with and robustness of the branches was determined with 1000 bootstrap replicates along with max-trees set at 1000 (Tamura *et al*, 2013) [29]. The number of replications inferred using the stopping criterion. Six different culture media namely, potato dextrose agar (PDA), carrot agar (CA), potato sucrose agar (PSA), Richard agar (RA), Honey peptone agar (HPA), Honey agar (HA) were used to assay the mycelial growth of the pathogen (Sikder *et al*, 2019) [25]. Different temperatures (15, 20, 25, 30 and 35°C) were maintained for the mycelial growth of the pathogen on PDA in an incubator. The mycelial growth was recorded at 7 days post inoculation (dpi) (Alam *et al*, 2010) [1]. The effect of light on the mycelial growth of the pathogen was by exposing the inoculated culture to 24 h light, 24 h dark and alternate cycle of 12 h light and 12 h dark in an environment chamber in which at room temperature (25 ± 2°C) was maintained (Singha *et al*, 2013) [27].

Mycelial growth inhibition of the pathogen was determined using duel culture technique in which biological control agent's namely-*Trichoderma reesei*, *Trichoderma harzianum*, and *Trichoderma asperellum* were used. A range of fungicides-Tilt 250 EC @ 100pppm, 250ppm, 500ppm; Amistar Top 325 SC @ 250ppm, 500ppm, and 750ppm were used against the pathogen under in vitro condition by poison food technique. PDA plates contains without any fungicides served as control. The percent growth inhibition of the fungus was calculated at 7 days post incubation (dpi). Data on effects of media, light, and temperature on mycelial growth of the studied fungi was analyzed using one-way ANOVA in SPSS-14. Mycelial growth inhibition was determined using formula, inhibition (%) = (C-T) × 100/C, Here, C indicate mycelial growth on control plates, T indicate mycelial growth on treated plates.

3. Results and Discussion

Aloe vera plants infected with fungus showed brownish color, rough, large spots were present on the surfaces of the leaves (Plate 1A). The fungus was greyish to dark in colour with a lighter border, the colonies covered by short, greyish, aerial hyphae on PDA medium (Plate 1B). Reverse side of the Petriplate dark brown or nearly black. A dark olivaceous fungus with profuse golden brown, branched and septet hyphae. Conidiophore short, unbranched, septet with one or few conidial scars. Conidia are obclavate to ellipsoidal, brown, with a short beak, transverse and longitudinal or obliquely septet, arising in mostly unbranched chains of ten or more (Plate 1C). Based on these phenotypic traits, the fungus seems to be *A. alternata*. Molecular characterization could ensure its position in the classification of the fungus. In Bangladesh, there was no record of fungal pathogen-*Alternaria alternata* causing leaf spot disease on *Aloe vera*.

The sequence alignment comprised 31 taxa of representative species of Pleosporaceae, including our taxa- *Alternaria alternata* (MH368103.1). In Blast search, present studied organism showed 99% identity with KJ739870.1 (*A. alternata*), KY859403.1 (*A. alternata*), KJ739874.1 (*A. alternata*), AY433814.1 (*A. alternata*) and GQ169728.1 (*A. alternata*). Phylogenetic tree was generated by Maximum likelihood (ML) with previously submitted ITS sequence in NCBI by other people around the world (Figure 1). In ML tree, Clade-I consists of eight taxa under the genus-*Alternaria* with 96% bootstrap value within *A. alternata*, this clade rooted with *Alternaria cinerariae*. Second clade had seven taxa, which includes seven different species of *Alternaria*. Clade-III had cluster of six taxa with five different species of *Alternaria*. Clade-IV consists of several taxa in which *Alternaria dauci* form sister clade with 99% bootstrap support. *Alternaria porri* and *Alternaria tomatophila* did not form any cluster in the phylogenetic tree. This phylogenetic tree demonstrates the position of *A. alternata* under the family-Pleosporaceae. ITS sequences are genetically constant or show little variation within species but vary between species within genus (Alam *et al*, 2009) [2].

The effect of different culture media *viz*., potato dextrose agar (PDA), carrot agar (CA), potato sucrose agar (PSA), Richard agar (RA), honey peptone agar (HPA), honey agar (HA) on mycelial growth of *A. alternata* and the statistical differences among the culture media have been presented in Figure 2. The results showed that the highest mycelial growth of *A. alternata* was recorded on PDA medium, which was followed by CA medium and the lowest growth was measured in HA medium (Figure 2 and Plate 2). The colour of the colony was dark brown with light margin on PDA, CA and RA media, light greyish on PSA medium, brown on HPA, and greyish on HA medium. Hubballi *et al*, (2010) [12] support the current results of *A. alternata* who reported PDA as one of the best media for the mycelial growth of *A. alternata* causing leaf blight of Noni (*Morinda citrifolia* L.). Koley and Mahapatra (2015) [14] cited that PDA media gave profuse mycelial growth *Alternaria solani* causing tomato early blight. Likewise, PDA was optimal medial for vegetative growth of *Alternaria carthami* causing leaf blight of safflower Awadhuya (1991) [4].

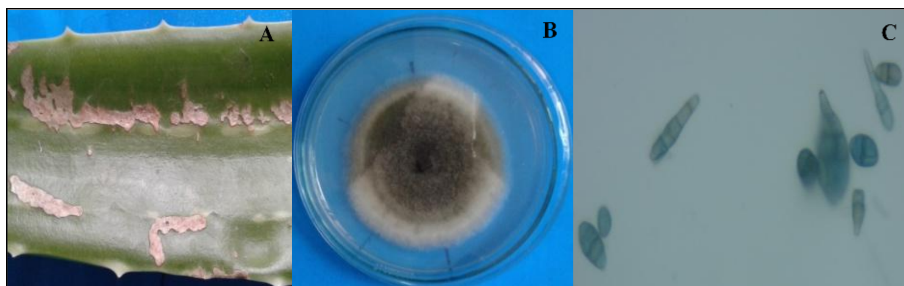

Plate 1: Leaf spot disease of *Aloe vera*. A: Leaf spot symptom of *Aloe vera*, B: Mycelial growth of *A. alternata* on PDA medium, C: Microscopic view of conidia (40 X 10x).

The effect of different light duration on mycelial growth of *A. alternata* have presented in Figure 3. The experimental plates were incubated at three different conditions viz., 24 hours Light, 24 hours Dark and 12/12 hours Light-Dark. We found *A. alternata* showed better mycelial growth under both complete dark condition and alternate 12/12h light-dark condition compared to continuous light condition (Figure 3). Hubballi *et al*, (2010) [12] who found alternate 12h/12h suitable for mycelial growth of *A. alternata*, support our results.

There is always an optimum temperature for the best growth of any fungus as temperature has a regulatory effect on fungal growth and development. Thus, present study investigated the effect of temperature on radial mycelial growth of *A. alternata* on PDA media under *in vitro* condition. The result revealed that the highest growth of *A. alternata* was recorded at 25℃, followed by 20℃ and least at 35℃ temperature (Figure 4). In our experiment is in consistent with the previous findings of Somappa *et al*, (2013) [28] who cited that the highest mycelia growth and sporulation of *A. solani* registered at 25^{0}C, a sudden fall in mycelial growth and sporulation observed at 30^{0}C and 35^{0}C; concluded that 25^{0}C is the optimum temperature for vegetative growth and sporulation of *A. solani*. Hubballi *et al*, (2010) [12] reported the suitable mycelial growth temperature between 25-30^{0}C for *A. alternata*. Garibaldi *et al*, (2007) [10] reported 27°C as an optimum temperature for the growth of *A. alternata*.

In the present study, biocontrol agents shown promising results in respect to restriction of the mycelial growth of the *A. alternata*. *Trichoderma reesei* gave the maximum inhibition of *A. alternata*, followed by *Trichodera harzianum* (Figure 5 and Plate 4A-C).

Several *Trichoderma* strains was evaluated under field conditions to assay their efficacy in suppressing *Alternaria* fruit rot disease and promoting chili plant growth in Bangladesh. Application of *Trichoderma harzianum* IMI 392432 significantly suppressed the disease caused *Alternaria tenuis* and improved both growth and yield (Begum *et al*, 2010) [6].

Zafar *et al*, (2013) [33] tested five *Trichoderma* species against *A. solani,* in which *Trichoderma viride and Trichoderma harzianum* most strongly suppressed the growth of *A. solani*. Rani *et al*, (2017) [21] evaluated several bioagents against *A. solani* in which, *Trichoderma harzianum* showed maximum growth inhibition of the pathogen and appeared to be the most effective control measures. Rahman *et al*, (2015) [20] reported *Trichoderma viride* as an effective biofungicides against *Alternaria porri*. Therefore, biocontrol agents seems potential to restrict mycelial growth of *A. alternata*.

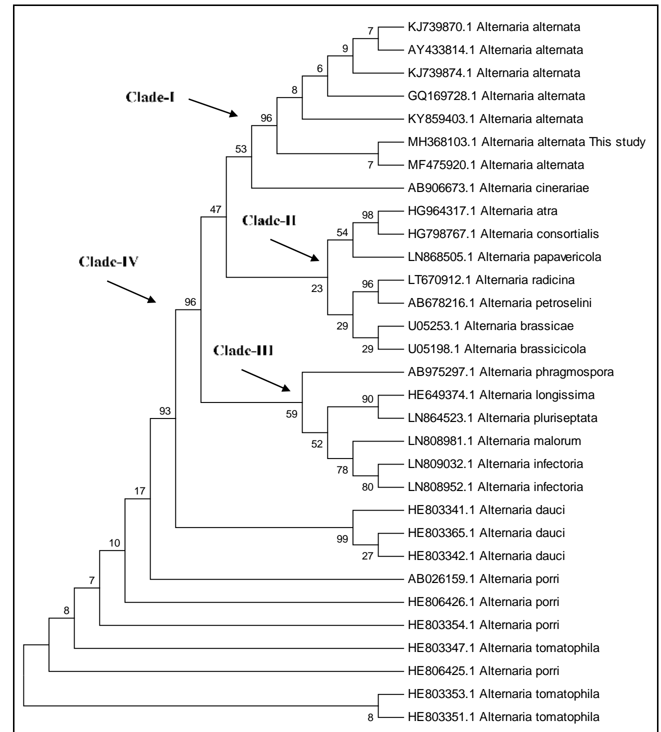

Fig 1: Maximum likelihood tree derived from analysis of ITS sequence dataset of the studied organism with bootstrap value (Bootstrap replication=1000). Our organism (MH368103.1) marked with 'This study'.

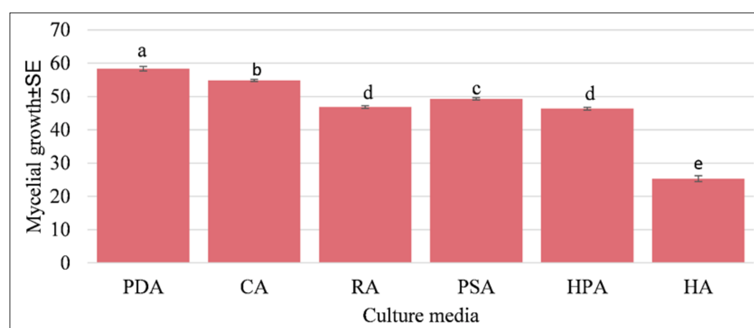

Fig 2: Effect of culture media on mycelial growth (mm) of *A. alternata* at 7 dpi. Data represents mean ± standard error of six replications, at 5% level of significance. PDA- potato dextrose agar, CA- carrot agar, PSA- potato sucrose agar, RA- Richard agar, HPA- honey peptone agar, HA- honey agar. Letter (s) on error bars are based on statistical analysis (Duncan's multiple range test) after one-way ANOVA. Means followed by the same letter(s) do not differ significantly ($p > 0.05$).

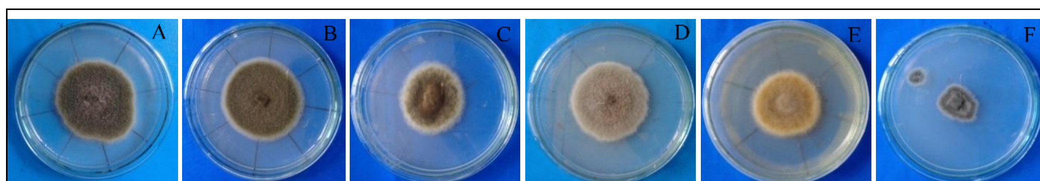

Plate 2: Mycelial growth of *A. alternata* on culture media at 25°C; A: PDA, B: CA, C: RA, D: PSA, E: HPA and F: HA medium.

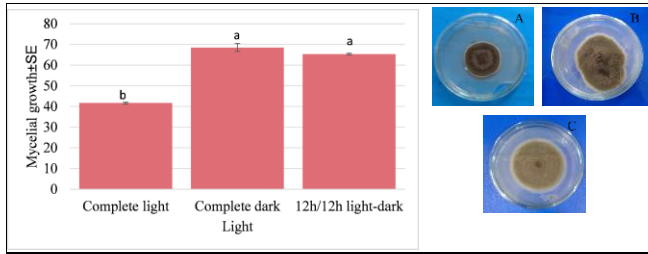

Fig 3: Effect of light regimes on mycelial growth (mm) of *A. alternata* 25°C at 7 dpi. Value represents as mean ± standard error of six replications. A. 24 h continuous light condition, B: 24 h continuous dark condition and C: Alternate 12 h/12h light-dark conditions. Letter (s) on error bars are based on statistical analysis (Duncan's multiple range test) after one-way ANOVA. Means followed by the same letter(s) do not differ significantly ($p > 0.05$)

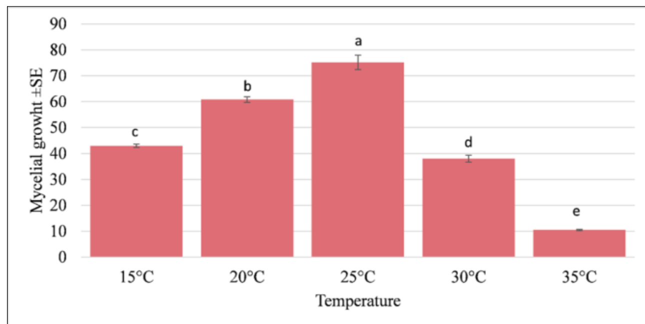

Fig 4: Effect of temperature on mycelial growth (mm) of *A. alternata* at 7 dpi. Value represents as mean ± standard error of six replications. Letter (s) on error bars are based on statistical analysis (Duncan's multiple range test) after one-way ANOVA. Means followed by the same letter(s) do not differ significantly ($p > 0.05$)

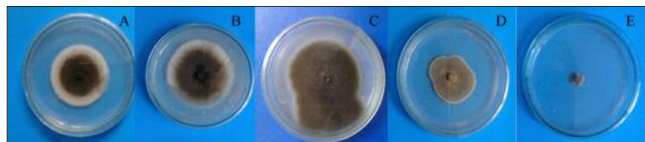

Plate 3: Mycelial growth of *A. alternata* on PDA media at different temperatures condition; A: Growth at 15°C; B: at 20°C; C: at 25°C; D: at 30°C and E: at 35°C temperature.

In the current investigation, the mycelial growth inhibition was sharply increased with increasing doses of Tilt 250EC (chemical name: Propiconazole) fungicides in which completely inhibition of the studied fungus recorded by the higher dose of the fungicide, followed by above 80% inhibition due to 250ppm concentration of this fungicide (Figure 5 and Plate 4G-I). Besides, the higher does (750 ppm) of Amistar (chemical name: Azoxystrobin) was able to restrict above 80% mycelial inhibition of *A. alternata*, which was close to 80% inhibition by 500 ppm of Amistar (Figure 5 and Plate 4D-F). Several earlier researchers support our present findings. Thejakumar and Devappa (2016) [30] cited that propiconazole at all concentration viz., 500, 1000 and 2000 ppm was showed complete inhibition of vegetative growth of *A. alternata* causing leaf spot disease of chilli. Kumar *et al*, (2017) [15] found two azole fungicides (Tilt: Propiconazole and Folicur: Tebuconazole) most effectively at concentration of 10ppm against *A. alternata* compared to other tested fungicides (Natio: Tebuconazole+ Trifloxystrobin, Bavistin: Carbendazim, Dithane Z-78: zineb and SAAF: Carbendazim+Mancozeb). Reuveni and Sheglov (2002) [22] mentioned that azoles fungicides was effective control of *A. alternata* pathogen in apple fruit crop.

Archana and Jamadar (2014) [3] also studied several fungicides against *A. alternata* and found that azole (propiconazole) reduced more disease incidence as compared to strobilurin and other fungicides. Singh and Majumdar (2002) [26] reported that propiconazole was the most suitable fungicide in controlling *A. alternata*. Gorawar *et al,* (2006) [11] observed that propiconazole, penconazole and hexaconazole at all three concentrations (0.025%, 0.05% and 0.1%) showed 100% inhibition of mycelial growth of *A. alternata*. Phapale *et al,* (2010) [19] reported propiconazole showed 100% reduction of *A. alternata* at 250, 500 and 1000ppm concentrations. Recently, Yadav *et al*, (2020) [32] reported that propiconazole as an effective fungicide in management of *A. alternata* causing leaf blight of tomato under both *in vitro* and field conditions. Kaur *et al*, (2020) [13] cited that two sprays of azoxystrobin (0.1%) in mid-January and mid-February were better in controlling the disease of Ber black fruit spot causing by *A. alternata*. Mazur *et al*, (2005) [17] also concluded that azoxystrobin showed higher efficacy in controlling Alternaria blight of carrot. Together with previous findings, it seems both Tilt (propiconazole) and Amistar (azoxystrobin) fungicides effective against *A. alternata*.

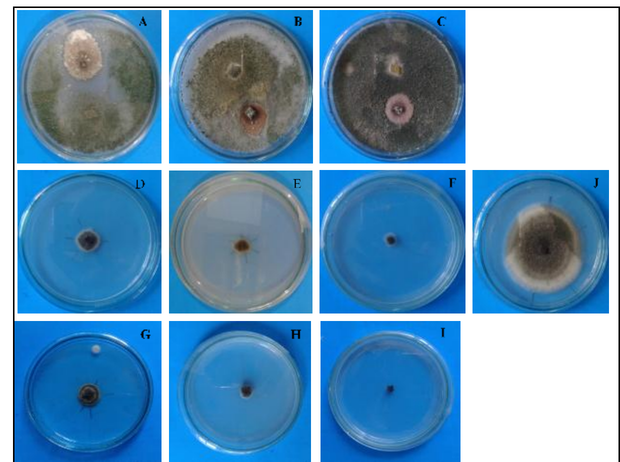

Plate 4: Effect of control measures on mycelial growth of *A. alternata* at 7 dpi. A: *Trichoderma asperellum* vs *A. alternata*, B: *Trichoderma harzianum* vs *A. alternata*, C: *Trichoderma reesei* vs *A. alternata*, D: Amistar (250ppm), E: Amistar (500ppm), F: Amistar (750ppm), G: Tilt (100ppm), H: Tilt (250ppm), I: Tilt (500ppm), J: Control.

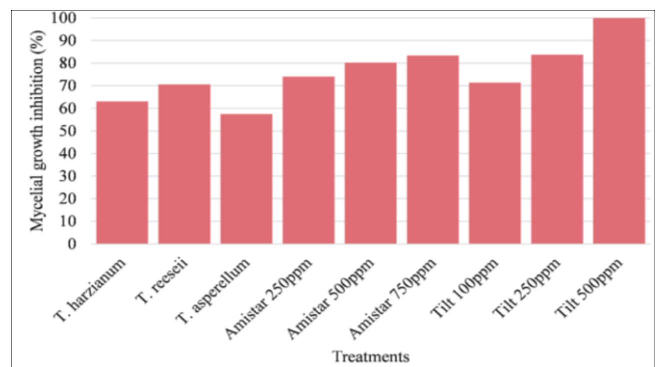

Fig 5: Effect of control measures on mycelial growth of *A. alternata* at 7 dpi. Value obtained from six replications.

4. Conclusions

Leaf spot disease of *Aloe vera* caused by *Alternaria alternata* in Bangladesh, a new record. Phenotypic traits and

molecular characterization confirmed the fungal identity. Phylogenetic tree was generated with ITS sequence data analysis by Maximum Likelihood demonstrates the position of *A. alternata* under the family-Pleosporaceae. Subsequently, the effects of different culture media, light and temperature on the fungal nourishment was evaluated. We also explored how to inhibit the fungal growth using biocontrol agents and commercial fungicides under *in vitro* condition. This study could be basis for field trial to find out efficacy of the tested biocontrol agents and fungicides.

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