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# *First Report Of Leaf Spot Disease Of Aloe Vera Caused By Fusarium Oxyporum In Bangladesh*

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**Abstract- Aloe vera (L.) Burm.f. is one of the important medicinal plants and extensively cultivating in various region of Bangladesh. The present experiments were conducted to find out the fungal pathogen affiliated with the infected leaves of Aloe vera collected from potted nursery plants and leaf samples collected from Natore, Bangladesh. Leaf spot disease causing fungus-Fusarium oxysporum, was identified through morphological characterization based on colony, mycelium and conidial features as well as molecular characterization using internal transcribed spacer sequencing of rDNA. Typical leaf spots were reproduced by artificial inoculation of the isolated fungus. Growth characteristics of the fungal pathogen was assessed on different solid culture media i.e., Potato Dextrose Agar, Carrot Agar, Potato Sucrose Agar, Richard Agar, Honey Peptone Agar, Hansen's Agar; at different temperatures (15, 20, 25, 30, 35ºC) and on different photoperiodic conditions (24h light, 24h dark, 12h alternate light-dark). F. oxysporum showed the maximum mycelial growth on both RA and CA media at 25ºC under complete dark condition. Competent biocontrol agents exhibited the maximum 50% mycelial growth inhibition whereas synthetic fungicides-Tilt 250EC (500ppm) showed the maximum of over 85% inhibition of the vegetative growth of the studied fungus. To the best of our knowledge, leaf spot disease of Aloe vera caused by Fusarium oxysporum in Bangladesh, a new record.**

**Keywords: Medicinal plant, molecular characterization, fungal biology, biocontrol, fungicides**

## I. INTRODUCTION

*Aloe vera* (L.) Burm. is a succulent, duought resistant, herbaceous medicinal plant belongs to the family Liliaceae. It is extensively cultivated in tropical and sub-tropical zones for its enchanting medicinal, ornamental and comestic uses [1]. Now-a-days it has been cultivating in different part of Bangladesh and become the principal agricultural product to earn livelihood in the Northern part, especially Natore District.

However, *Aloe vera* is vulnerable to various fungal infestations. Some fungal pathogens and non-pathogens are known to produce mycotoxins that are hazardous to human and animal health, which can cause cancer, hemorrhage, edema and immune deficiency [2]. A number of fungal diseases on *Aloe vera* caused by *Fusarium* spp. have been reported, namely purple leaf spot caused by *Fusarium phyllophilum* [3], ring spot of *Aloe barbadensis* Mill. by *Haematonectria haematococca* (anamorph: *Fusarium* sp.) in Japan [4], leaf rot of *A. barbadensis* by *F. oxysporum* in Bali, Indonesia [5], leaf spot of *Aloe* by *F. oxysporum* and *Fusarium solani,* in Iraq [6], *Fusrium roseum* and *F. oxysporum, Fusarium proliferatum* in India [7], [8], *Fusarium* sp. in Bangladesh [9]. However, there is no record of *F. oxysporum* causing leaf spot disease on *Aloe vera* in Bangladesh. Therefore, the present experiments were conducted to detect and find out the causal organism of leaf spot of *Aloe vera*; to characterize the pathogen using morphological and molecular attributes; to study of fungal biology (culture media, temperature, light) of the pathogen; to evaluate the efficacy of antagonistic fungi, and chemical fungicides against the fungal pathogen.

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## II. Materials and Methods

### *2.1. Isolation and identification of the fungi*

*Aloe vera* leaves with characteristic disease symptoms were collected from Natore, Bangladesh. Samples were also collected from the experimental sites of Botanical Garden, JU, Savar, Dhaka, Bangladesh. Tissue planting method was employed to isolate fungal pathogen; subculture was maintained on PDA medium; the pure culture was stored in refrigerator at -4$^0$C. Isolated fungus was identified on the basis of colony morphology and conidial features [10]. *In vitro* pathogenicity test of the isolated fungus was conducted using detached leaf method.

For molecular characterization, fungal genomic DNA was extracted using DNA extraction Kit (Promega, Madison, USA). The primer ITS4 (5-TCCTCCGCTTATTGATATGC-3) and ITS5 (5-GGAAGTAAAAGTCGTAACAAGG-3) were used for amplification of the target region of the fungus [11]. The PCR reaction was performed in a 25µl reaction mixture, containing 5 µl DNA template (20ng/µl), 2.5µl of each of forward and reverse primer, 12.5µl GoTaq G2 Hot Start Green Master Mix (dNTPs, Buffer, $MgCl_2$, Taq Polymerase; Promega 2X, Madison, USA), and 2.5 µl water. The PCR cycles were performed with an 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, termination with a 10-minutes step at 72°C [12]. The Maxwell® 16 DNA Purification Kits were used to purify the PCR products (Promega, Madison, USA) and were sequenced in FIRST BASE Laboratories SdnBhd (Malaysia). Sequence data were submitted to NCBI and accession number was received subsequently.

A BLAST search with the internal transcribed spacer (ITS) sequences was used to reveal the closest matching taxa in family-Nectriaceae. Multiple sequence alignments were performed using MEGA6 [13], [14]. Maximum likelihood (ML), Neighbor-joining (NJ), and Maximum parsimony (MP) analysis was done with and robustness of the branches was determined with 1000 bootstrap replicates. The number of replications was deduced using the stopping criterion.

### *2.2. Effect of culture media and environmental factors on fungal growth*

Six different fungal culture media *viz.*, Potato Dextrose Agar (PDA), Potato Sucrose Agar (PSA), Carrot Agar (CA), Richard Agar (RA), Honey Peptone Agar (HPA) and Hansen's Agar (HA) were used to assay the mycelial growth of the fungal pathogen [15]. The effect of temperature on mycelial growth of the studied fungus was assessed as method described earlier [16]. Different temperatures regimes (15, 20, 25, 30 and 35°C) were maintained to observe the mycelial growth of the fungus on PDA medium in an incubator. The effect of photoperiod on the mycelial growth of the fungal pathogen was assessed by exposing the inoculated culture to 24h light, 24h dark and alternate cycle of 12h light and 12h dark in an environment chamber in which room temperature (25 ± 2°C) was maintained [17]. The mycelial growth regarding effect of culture media, light and temperature was recorded at 7 days post inoculation (dpi).

### *2.3.* In vitro *mycelia growth inhibition of the tested pathogen*

Mycelial growth inhibition of the fungal pathogen was determined using dual culture technique, in which three *Trichoderma* based bio-control agents were employed as described earlier [18]. Besides, two fungicides-Tilt 250 EC @ 100ppm, 250ppm, and 500ppm; Amistar Top 325 SC @ 250ppm, 500ppm, and 750ppm were also tested against the pathogen under *in vitro* condition by poison food technique [19]. PDA medium without any treatment served as control. The percent growth inhibition of the fungus was calculated at 7 dpi. Generated data were checked for normality and homogeneity of variance. Data on the effect of media, light, and temperature on the mycelial growth of *F. oxysporum* was found to be normal and analyzed using one-way ANOVA with Duncan's Post-Hoc test in SPSS-20.

## III. Results and Discussion

### *3.1. Identification and characterization of the fungus*

In *Aloe vera* plant, the fungal pathogen produced symptom with brown color margin and pale white color spots in the centre of leaf (Fig. 1A). These spots were inoculated on PDA medium and produced white color colony which later turned into pinkish color (Fig. 1B). In microscopic study, mycelium was hyaline and branched. Macroconidia were sickle-shaped, delicate and thin-walled, with 2-5 septation, which formed from monophialides on branched conidiophores (Fig. 1C). Microconidia were oval or kidney shaped, produced on monophialides. The fungus also formed a single, terminal chlamydospore (Fig. 1C). These morphological features indicated the fungus as *Fusarium* sp. However, these features did not confirm the identity of the fungus at species rank.

The pathogenic fungus associated with leaf spot disease of *Aloe vera* was further confirmed by pathogenicity test through detached leaf methods under *in vitro* condition and molecular characterization via ITS sequencing. The PCR yielded an amplicon size about 550bp during the amplification of the ITS region of *Fusarium* sp. In BLAST search, our organism showed 99 to 100% identity with different strain of *Fusarium oxysporum*. The sequences data was submitted to NCBI database and received the accession number (MH368097.1). The phylogenetic trees were produced by Maximum Likelihood (ML), Neighbour Joining (NJ), and Maximum Parsimony (MP). Topologies of all three phylogenetic trees were almost similar. We have presented MP tree in this manuscript only. In MP tree, Clade-IV consists of 9 species of *F. oxysporum* including present studied fungus with above 50% bootstrap value. Our organism has been marked with "This study". Considering the closeness of the species, tested fungus was confirmed its identity as *F. oxysporum*. *Fusarium solani* was characterized based on ITS sequence [20]. Likewise, it has also been reported that amplification of ITS region of *F. solani* yielded an amplicon of approximately 550 to 570 base pairs to identify the fungus [21].

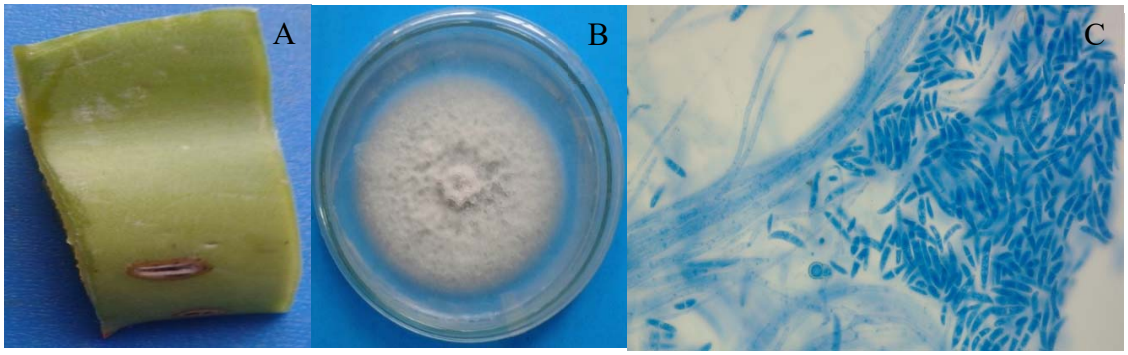


Fig. 1. Morphological characteristics of *F. oxysporum* causing leaf spot disease in *Aloe vera* plant. A: Leaf spot disease symptom, B: Vegetative growth of fungus on PDA medium, C: Microscopic view of conidia and conidiophore (40X).

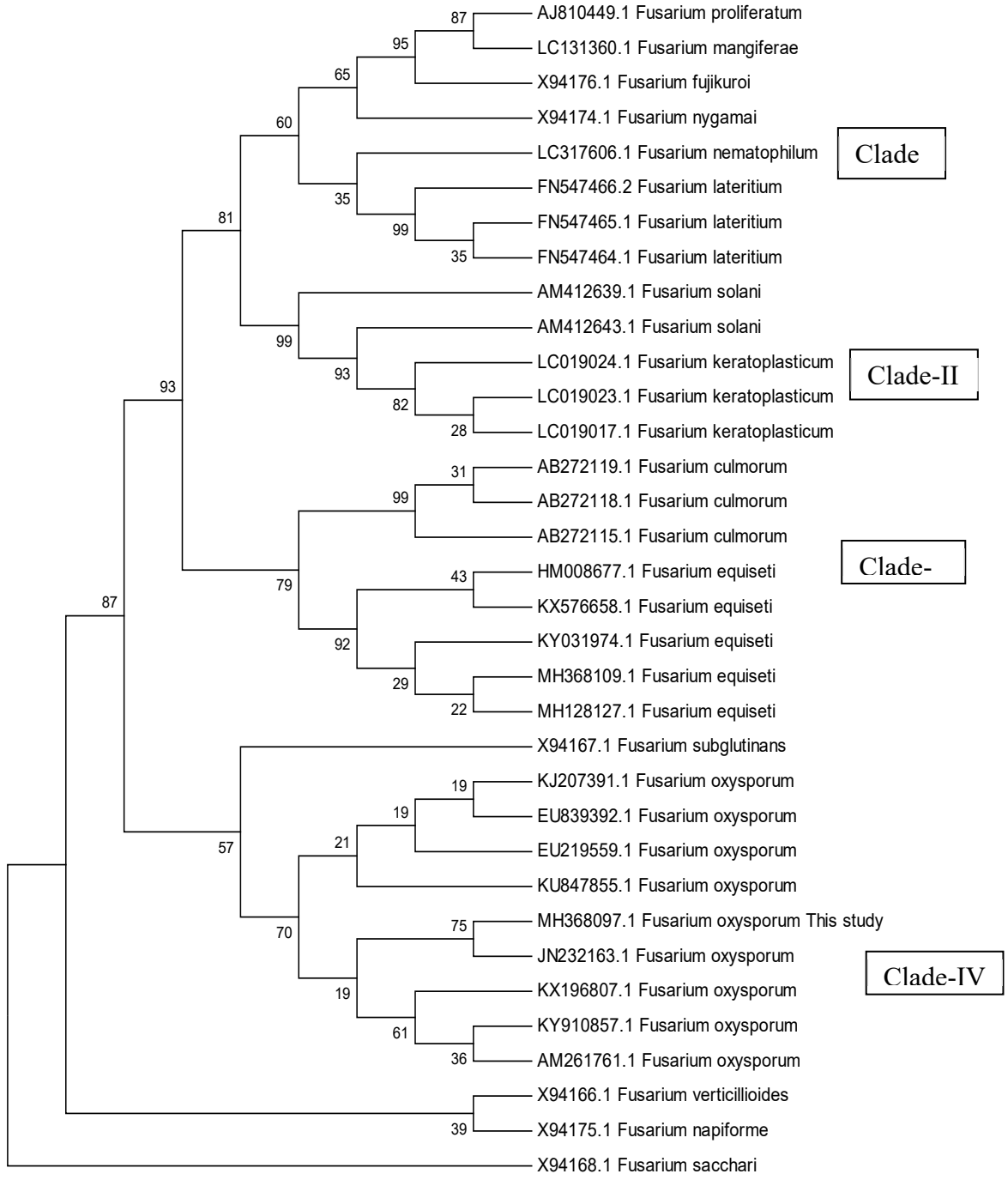


Fig. 2. Maximum parsimony tree of the 18S rDNA sequence of the studied organism with bootstrap value. Our organism (MH368097.1) marked with this study.

### *3.2. Growth characteristics of* F. oxysporum

The effect of six different solid culture media *viz.*, PDA, CA, RA, PSA, HPA and HA on the mycelial growth of *F. oxysporum* was evaluated (Fig. 3 and 4). The highest mycelial growth of *F. oxysporum* was recorded on RA medium, followed by CA medium while the minimum on HA medium. Although we did not observed the best mycelial growth of *F. oxysporum* on PDA, several report have been suggested PDA as suitable medium for this fungal growth [22], [23], [24]. In another study, the maximum growth of *F. oxysporum* f. sp. *gerberae* was recorded on Oat meal agar, followed by RA, Czapek's Dox agar and PDA [25].

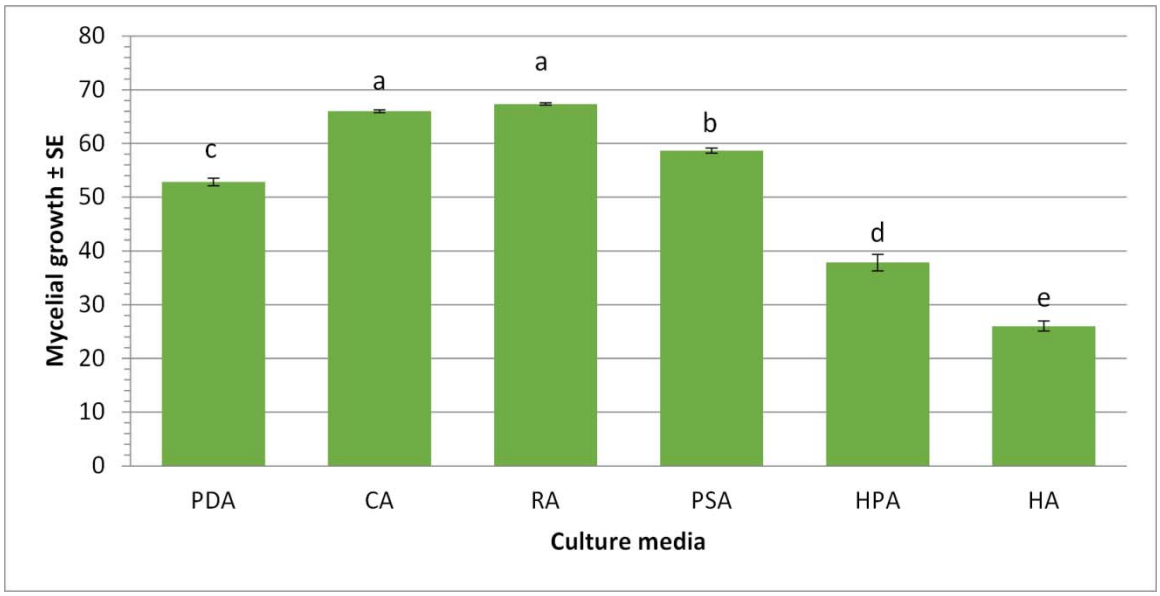


Fig. 3. Effect of culture media on the mycelial growth of *F. oxysporum* at 7 dpi. Data represents mean ± standard error (SE) of six replications. Means followed by a common letter (s) do not differ significantly at the 5% level by DMRT. Here, PDA- Potato Dextrose Agar, CA- Carrot Agar, PSA- Potato Sucrose Agar, RA- Richard Agar, HPA- Honey Peptone Agar, HA- Hansen's Agar.

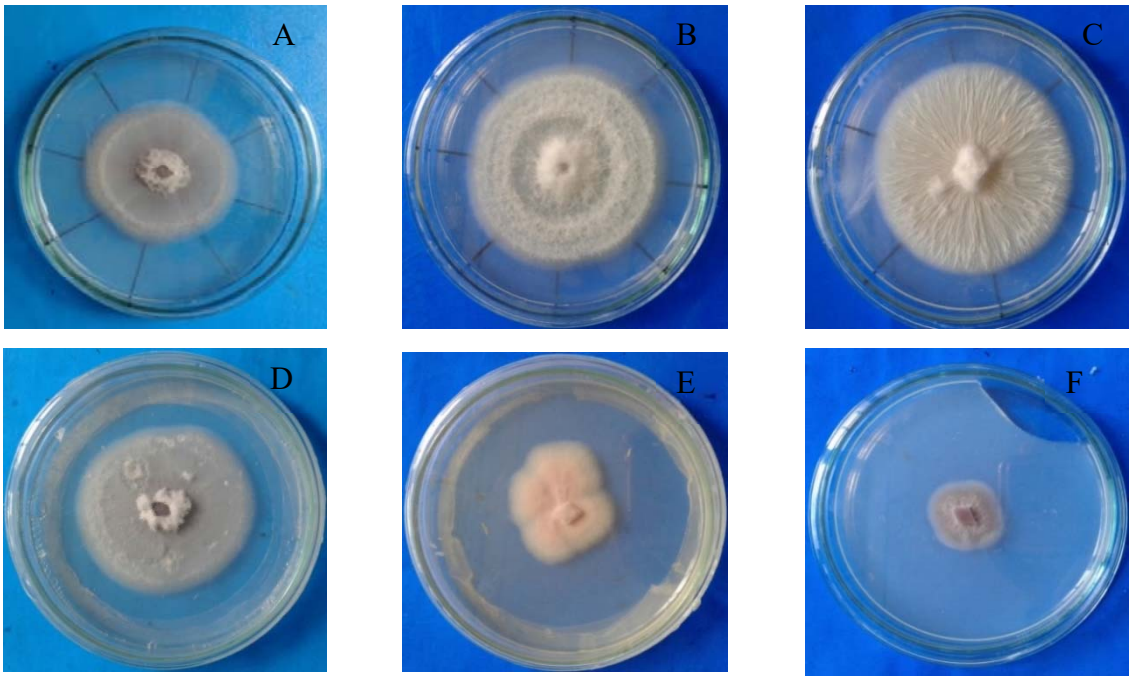


Fig. 4. Effect of culture media on mycelial growth of *F. oxysporum* at 7 dpi. A: Potato Dextrose Agar, B: Carrot Agar, C: Richard Agar, D: Potato Sucrose Agar, E: Honey Peptone Agar, F: Hansen's Agar.

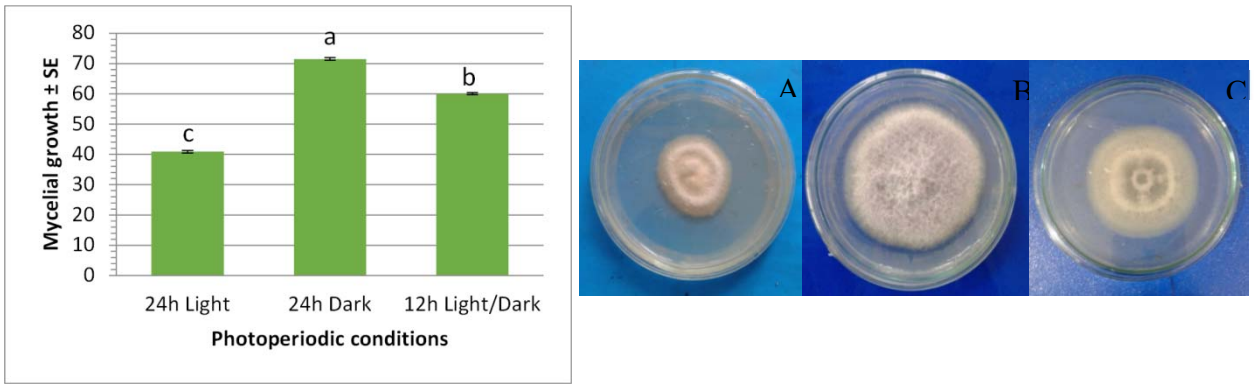


Fig. 5. Effect of light regimes on the mycelial growth of *F. oxysporum* at 7 dpi. Value represents as Mean± standard error (SE) of six replications. Means followed by a common letter (s) do not differ significantly at the 5% level by DMRT. A: 24h light, B: 24h dark and C: 12h light and 12h dark.

The effects of different photoperiodic conditions on the mycelial growth of the tested fungus have been presented in Fig. 5. The experimental plates were incubated at three different conditions *viz.*, 24h Light, 24h Dark and 12/12h Light-Dark. *F. oxysporum* fovoured complete dark condition, in which the outmost mycelial growth was recorded, followed by alternate light and dark condition and least due to continuous light condition. Our results are supported by a previous researcher who reported that the radial mycelial growths of all studied fungi (*F. solani, F. oxysporum, F. sacchari, F. proliferatum, and F. globosum*) were significantly reduced under complete light compared to complete dark condition [26]. However, another study reported the highest mycelial growth of *Fusarium oxysporum* f.sp. *cubense* under 12/12h light and darkness, followed by continuous light while lowest growth of the fungus was observed under continuous dark conditions [27]. We speculate that this kind of variation could be due to different isolates of the fungus.

There is always an optimum temperature for the best growth of any fungus as temperature has a regulatory effect on fungal growth and development. Hence, we investigated the effect of five different temperatures on radial mycelial growth of *F. oxysporum* on PDA media under *in vitro* condition (Fig. 6 and 7). Data revealed that there was an increasing trend of mycelial growth of *F. oxysporum* up to 25°C, afterwards started to decline. The utmost vegetative growth was recorded at $25^0$C, followed by 30°C, 20°C, the lowest growth was found at both extreme temperatures (15°C and 35°C). The results are supported by previous study in which, maximum mycelial growth of *F. oxysporum* f. sp. *lactucae* was observed at 25ºC [28]. Similarly, the most favorable temperature range for growth and sporulation of *Fusarium oxysporum* was obtained between 25 to 30°C [29], [30]. Another studyconcluded that temperature at $22^0$ C or above was found as most suitable for *F. oxysporum* [31].

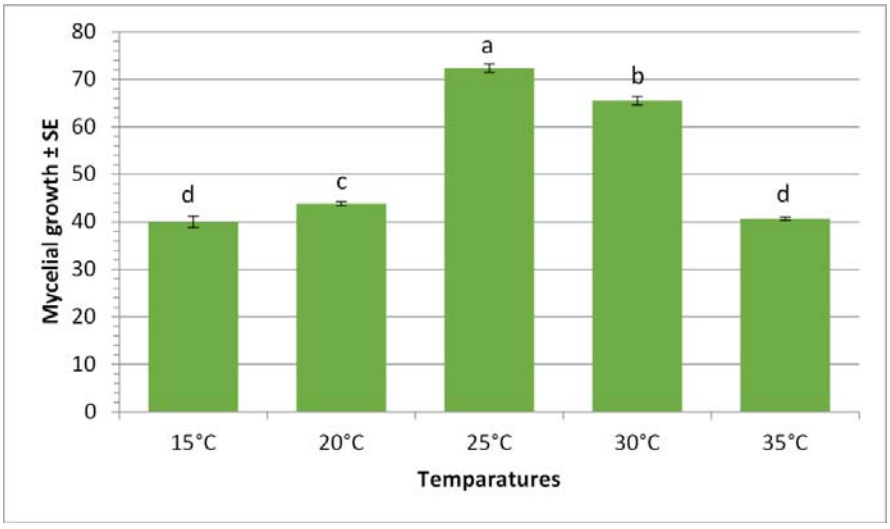


Fig. 6. Effect of temperature on mycelial growth of *F. oxysporum* at 7 dpi. Value represents as mean ± standard error (SE) of six replications. Means followed by a common letter (s) do not differ significantly at the 5% level by DMRT.

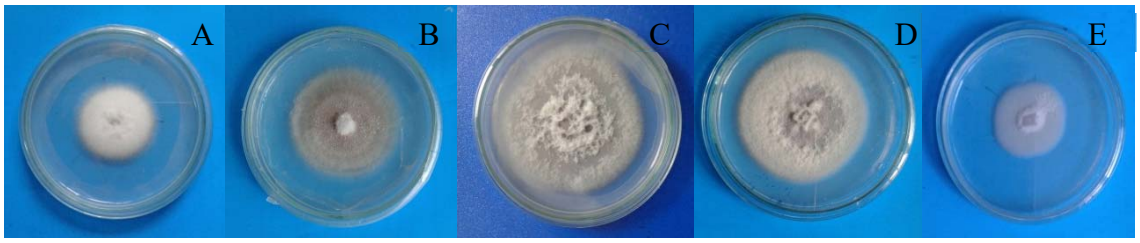


Fig. 7. Effect of temperature on mycelial growth of *F. oxysporum* at 7 dpi. A: 15°C; B: 20°C; C: 25°C; D: 30°C; and E: 35°C.

### *3.3. Effect of biological control agents on* F. oxysporum

In the present study, different bio-control agents showed a varying degree of mycelial growth inhibition. Fungal bio-control agents-*Trichoderma reesei* and *T. asperellum* showed similar degree of antagonism to the fungal pathogen-*F. oxysporum.* Both *T. reesei* and *T. asperellum* exhibited about 50% mycelial growth inhibition (Fig. 8 and 9), while *T. harzianum* showed below 50% inhibition of vegetative growth of *F. oxysporum*. These findings are supported by other group of scientists who cited that *Trichoderma* based biocontrol agents showed the utmost mycelial inhibition of *F. oxysporum* [32]. Likewise, *Trichoderma* were reported to be promising in suppressing disease incidence caused by *Fusarium oxysporum* [33]; and *Fusarium solani* [34]. Furthermore, the effectiveness of *Trichoderma* spp. (*T. harzianum*, *T. viride, T. koningii*- green strain and *T. viride*-yellow strain) was proven against *Fusarium solani* f. sp. *melongenae* under both laboratory and pot conditions [18]. *Trichoderma harzianum* was reported as antagonistic to *Fusarium oxysporum* [35], [36]. Besides, biological antagonistic agent *Trichoderma asperellum* was shown preventive activity against *Fusarium oxysporum* [37].

### *3.4. Effect of fungicides on mycelial growth of* F. oxysporum

The mycelial growth inhibition of *F. oxysporum* was found to be increased gradually with increasing concentration (250ppm to 750ppm) of Amistar top (Fig. 8 and 9). The maximum mycelial inhibition (81%) of the fungus was recorded due to 750ppm of Amistar top, followed by 64% at 500ppm while lowest 56% at 250ppm. Importantly, fungicide-Tilt 250EC (active ingredient: propiconazole) exhibited the better mycelial inhibition against *F. oxysporum*. The mycelial growth inhibition of the fungus was varied from 75% to 85% due to Tilt 250EC (Fig. 8 and 9). The maximum vegetative growth inhibition was recorded at 500ppm of Tilt 250EC. Similar results were found in another *in vitro* study, in which Tall 25 EC (active ingredient: propiconazole) completely inhibited the radial growth of *Fusarium moniliforme* at 200-500ppm concentrations. Besides, Propiconazole + Prochloraz combination was shown promising inhibitory activity against *Fusarium* sp. [36]. Another study revealed the efficiency of fungicides- Propiconazole and Azoxystrobin against *F. oxysporum* [39].

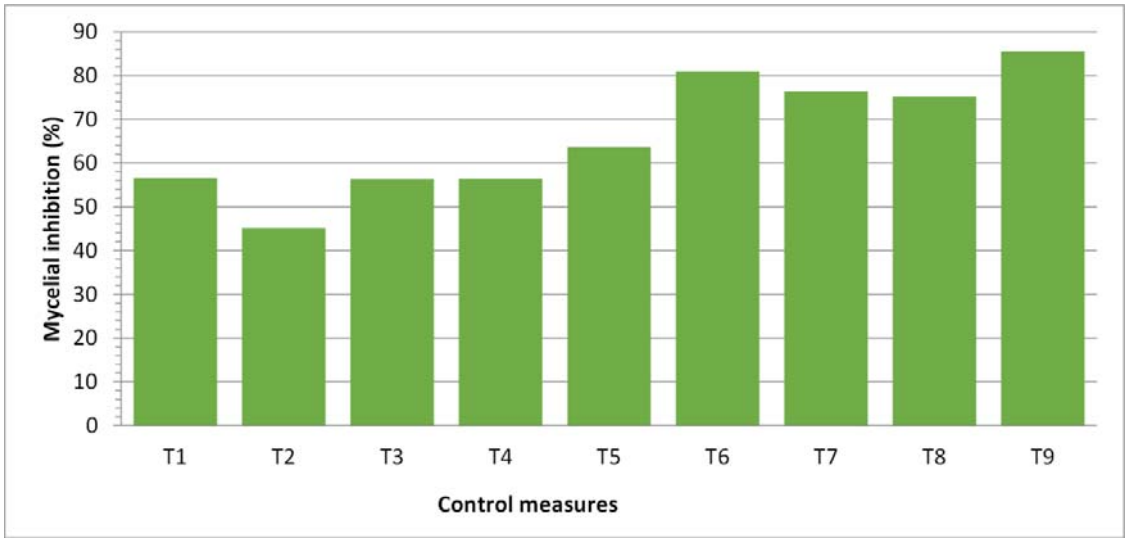


Fig. 8. Effect of biocontrol agents and fungicides on mycelial growth of *Fusarium oxysporum* at 7 dpi. (Here, T1-*Trichoderma asperellum*, T2- *T. harzianum*, T3- *T. reesei*, T4- Amister top (250ppm), T5- Amister top (500ppm), T6- Amister top (750ppm), T7- Tilt (100ppm), T8- Tilt (250ppm) and T9- Tilt (500ppm)).

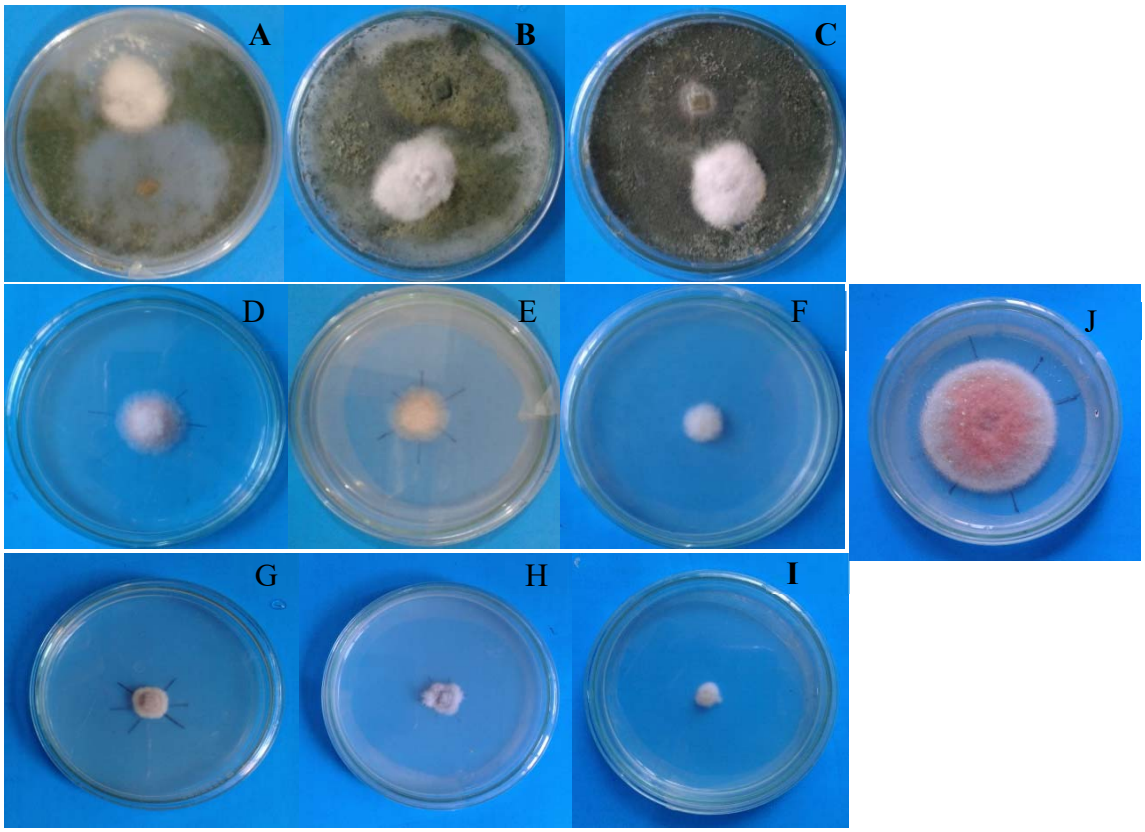


Fig. 9. Effect of bio-control agents and fungicides on mycelial growth of *F. oxysporum* at 7 dpi. A: *Trichoderma asperellum* vs. *F. oxysporum*; B: *T. harzianum* vs. *F. oxysporum*; C: *T. reesei* vs. *F. oxysporum*; D: Amistar (250ppm); E: Amistar (500ppm); F: Amistar (750ppm); G: Tilt (100ppm); H: Tilt (250ppm); I: Tilt (500ppm); J: Control.

## IV. Conclusion

Due to succulent nature of *A. vera* plant, they are vulnearable to pathogen attack. We identified and characterized the leaf spot disease causing fungus *F. Oxysporum*. Besides, trial on culture media, light and temperature of *F. oxysporum* revealed a great regulatory effect on the fungal growth. Preliminary *in vitro* studies showed that biological control agents and synthetic fungicides could be used to manage the leaf spot disease of *A. vera*. However, further pot and field trial is required to confirm the inhibitory effect of antagonists and fungicides against the fungus.

## V. Acknowlwdgment

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