Jahangirnagar University J. Biol. Sci. **11**(1 & 2): 1-11, 2022 (June & December)

Molecular characterization of endophytic fungi-*Daldinia eschscholtzii* from *Aloe vera* plants in Bangladesh

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Abstract

Aloe vera (L.) Burm. f. is a plant of health, beauty and medicine that is widely used in Bangladesh with high demand. An experiment was performed to find out the association of endophytic fungus with *Aloe vera* leaves and to assess their growth behavior on different culture media, temperatures and pH. The endophytic fungus-*Daldinia eschscholtzii* (Ehrenb.) Rehm. was identified based on the morphological and molecular characterization using the ITS-rDNA sequence. Various solid culture media were used to assess the fungal growth, and temperature regimes i.e., 15℃, 20℃, 25℃, 30℃, 35℃ were investigated. The fungal endophyte showed maximum mycelial growth on Potato Sucrose Agar followed by Potato Dextrose Agar and Carrot Agar media. Optimum growth and development of the endophyte was recorded at temperature 25 to 35℃. We observed the effect of five different pH conditions, *viz*., 5, 6, 7, 8, 9 on the mycelial growth of the fungus. However, the fungal endophyte was very insensitive to the pH of the substrate. To the best of our knowledge, this is the first known occurrence of *D. eschscholtzii* as an endophyte in *Aloe vera* in Bangladesh.

Key words: Endophyte, medicinal plant, culture media, temperature, pH.

INTRODUCTION

The plant *Aloe vera* (L.) Burm. belongs to the Liliaceae family and has been used for centuries for its medicinal, health, beauty and skin care properties. Today, this miracle plant is used for various purposes of dermatological treatment. This was known to the Egyptians as "the herb of immortality" (Surjushe *et al*., 2008). It has become the main livelihood crop in Natore and as well as other districts of Bangladesh (Shahidullah & Haque, 2010). The ability of endophytic fungi (endophytes) to produce plant-specific chemicals is attracting the interest of scientists constantly. The creation of a symbiotic relationship between the host plant and its endophytes can result from long-term interactions between the host plant and endophytes. Through this relationship, metabolites can be exchange between the plant and the endophytes (Ameen *et al*., 2021). Endophytic fungi are known to produce metabolites such as alkaloids, flavonoids, terpenoids, steroids, phenols, phenolic acids, quinones, peptides and isocoumarin derivatives (Zhang *et al*., 2006). *Aloe vera* is an excellent model plant for stydying how colonization by fungal endophyte affects secondary metabolism.

They have significant economic implications, a well-studied chemical profile, and it is known that colonization of endophytic fungi can modify certain of their medicinal compounds (Rahul *et al*., 2015; Lata *et al*., 2003). Because of the *Aloe* plants are used in medicine, cosmetics, juice preparation and other health issues studying of fungal

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endophytes associated with *Aloe vera* leaves is of public interest. Mycotoxins are produced by some fungi, both pathogenic and others that infect their hosts. Health risks from mycotoxins exist for both humans and animals which can cause cancer, bleeding, edema and immunodeficiency (Ahmmed *et al*., 2020). But endophytic fungi increase metabolic activities and accumulate numerous secondary metabolites during the symbiosis with the plant. In Saudi Arabia, Ameen *et al*. (2021) studied the endophytic fungus of *Aloe vera* through isolation, identification, and bioactivity analysis collected in the Asir desert. Rahul *et al*. (2015) performed an experiment on the antifungal and enzymatic activity of endophytic fungi associated with *Aloe vera*. A total of 18 endophytic fungi have been identified from the leaves, stems and roots of *Aloe vera*. Yadav *et al*. (2016) carried out a further study on plant growth-promoting properties and phytochemical analyses of fungal endophyte of *Aloe vera*.

Three endophytic fungi showed growth-promoting properties such as indole acetic acid (IAA) production, phosphate solubilization, and five endophytes were found to be siderophore positive. Three fungal isolates were active in producing ammonia, but none of the isolates showed activity in producing hydrogen cyanide (HCN). Chowdhary & Sharma (2020) investigated on the potential of fungal endophytes in the inflorescence of *Aloe vera* to promote plant growth and act as biocontrol agents. In India, Mane & Vedamurthy (2020) reported the physiology of *Aspergillus nomius* EF8-RSM found as an endophyte in *Aloe vera* plant. However, there is no adequate data on the association of endophtic fungi with the *Aloe vera* plant in Bangladesh. Therefore, the current investigation was aimed to isolate and identify the endophytic fungi of *Aloe vera* plant and to evaluate the effects of culture media, temperature regime and pH conditions on the growth and development of the fungus *in vitro*.

MATERIALS AND METHODS

Collection of plant sample: A fresh part of the *Aloe vera* plant was used as the material for the present experiment. The sample was collected from the potted nursery plant in the experimental site of Botanical Garden, Department of Botany, Jahangirnagar University (JU), Savar, Dhaka and also in the *Aloe vera* growing areas in Natore District, Bangladesh. The sample was collected in a zippered poly bag to avoid the external contamination. The laboratory part of the experiment was performed at Mycology and Plant Pathology Laboratory, Department of Botany, Faculty of Biological Sciences, Jahangirnagar University (JU), Savar, Dhaka-1342. Part of the molecular work was carried out in the Department of Biotechnology and Genetic Engineering, JU.

Isolation and identification of fungal endophyte: The collected plant samples were immediately transported aseptically to the laboratory and washed thoroughly under running tap water to remove the dust particles. The plant samples were then cut into 2 mm long pieces and immersed in 5% NaOCl for 3 minutes to allow surface sterilization. Samples were air dried in the laminar air flow booth after immediately cleaning with distilled water to remove NaOCl. The outer part of the plant samples was removed and the inner parts were collected and inoculated into a petri dish with potato dextrose agar (PDA) medium with 10% lactic acid and incubated at 25°C (Ahmmed *et al*., 2022). After

3 days, the fungal hyphae were evident on the sample and the incubated agar plates were examined for endophytic fungi. In addition, carefully selected hyphal ends from morphologically recognized endophytic fungi were transferred to freshly prepared potato dextrose agar medium. After 3 dpi (days post-incubation), a pure culture of the endophytic fungus was found and the culture was stored at -4℃ in the refrigerator for long-term uses.

Isolated fungal endophyte from the fresh tissues of *A. vera* leaf samples was identified on the basis of colony color, colony appearance, conidial structure and pigmentation, growth rate and arrangement of conidiophores under microscopic observation as reported by Ju *et al*. (1997). For molecular characterization, fungal genomic DNA was extracted from the freshly cultivated mycelia on PDA medium using the DNA extraction kit (Promega, USA). A nanodrop spectrophotometer (ND2000, Thermo Scientific, USA) was used to determine DNA concentration. Polymerase chain reaction (PCR) was performed using primers ITS4 and ITS5 for fungal DNA amplification (White *et al*., 1990). A LA Taq (TAKARA BIO INC, Japan) in a 25 L reaction mixture, the PCR reaction was carried out using 20 ng of genomic DNA as a template. Taq polymerase was activated at 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 5 minutes each, with a 10-minute step at 72° C to complete the process (Mallik *et al*., 2021). The amplified products were purified using Maxwell 16 DNA purification kits (Promega, USA). Approximately 650 bp purified PCR products were sequenced using 2 primers at FIRST BASE Laboratories SdnBhd (Kuala Lumpur, Malaysia). After trimming low quality named bases (Phred score < 20) at both ends and processing, the sequence data was submitted to the NCBI GenBank and assigned an accession number. A BLAST search was performed of the ITS sequences to determine the taxa that are closely matched to the family Xylariaceae. Multiple sequence alignments and phylogenetic tree were constructed from MEGA6 (Tamura *et al*., 2013).

Evaluation of the effect of culture media, temperature and pH on the vegetative growth of the endophyte: Different media components affect the growth and development of the fungi. In the current study, six separate culture media, *viz*., Potato Dextrose Agar, Honey Peptone Agar, Potato Sucrose Agar, Richard Agar, Carrot Agar and Hansen's Agar, used for *in vitro* assessment of fungal endophyte mycelial growth (Ahmmed *et al*., 2021). Actively growing endophytic fungus on the PDA medium was cut into 2 mm diameter discs and inoculated into the center of the tested medium. The inoculated plates containing different media were kept in an incubator at 25°C. Radial vegetative growth data were recorded after 7 days of incubation. Temperature is an important parameter to study the biological activity of fungi. The influence of temperature on the mycelium growth of the examined fungus was calculated according to Sultana *et al*. (2022). Five individual temperature conditions, i.e., 15°C, 20°C, 25°C, 30°C and 35°C were maintained for *in vitro* evaluation of mycelial growth of the fungal isolate on PDA medium. Inoculation techniques previously described were used for this experiment. All experiments were carried out in the BOD incubator (model: POL-EKO, APARATURA). Radial growth data were recorded at 7 days post incubation (dpi). Fungal growth is typically quite sensitive to different pH values. It is a crucial research factor in studying the biological activities of fungi. Five specific pH values, namely 5, 6, 7, 8 and 9, were

evaluated during the experiment on PDA medium. The pH was adjusted by adding 1N HCl and 1N NaOH solution. Radial growth data were recorded 7 days after incubation (Sikder *et al*., 2020).

Statistical analysis: The data collected during the experiments were examined for normality and homogeneity of variance. The effect of culture media and temperature regime on the studied vegetative fungal growth was found to be normal and determined by one-way ANOVA analysis and Duncan's post hoc test in SPSS-20. The molecular study with ITS rDNA sequence data was performed using BLASTn tools and the MEGA-X software.

RESULTS AND DISCUSSION

Morphological identification: The fungal endophyte was identified using standard protocols. On PDA medium, the fungus showed a pale whitish color, and when mature, it showed a light brown color at the center of the colony (Fig. 1A). On microscopic examination, mycelium was septate and branched, thin-walled, hyaline to brown colored (melanized), conidiophores septate, hyaline to brown, mononematous, dichotomous or trichotomous irregularly branched with 1–3 conidiogenous cells at its end, conidiogenous cells are hyaline, cylindrical shape containing hyaline conidia unicellular and circular to oval, ellipsoidal in shape and solitary (Fig. 1B). The colony turned a smoky gray color with a slight olive tinge with age when the mycelium was fully differentiated and also indicated the sporulation of the fungus. The fungus appeared on the opposite side of the Petri dish with a black-grey coloration. The primary identification of the fungus as *Daldinia eschscholtzii* was supported by morphological characters.

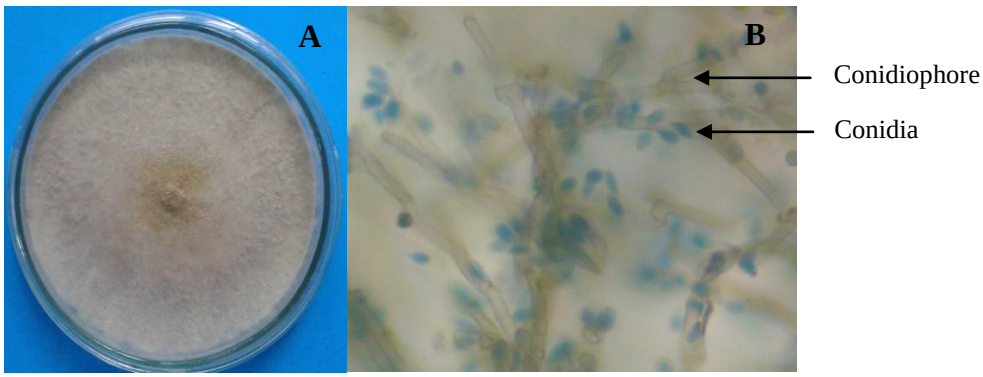

Fig. 1. Morphological characteristics of the endophytic fungus *Daldinia eschscholtzii* associated with *Aloe vera* leaves. A: Vegetative growth on PDA medium, B: Micrograph of conidia and conidiophore (40×10X)

Similar work was done in Thailand by Samarakoon *et al*. (2019), the *Daldinia eschscholtzii* as endophytes from the leaves of *Musa* sp. They described that *D. eschscholtzii* forms gray color colonies with olive green spots that later turn to dingy green. At maturity, the center of the colony begins to turn purple. The entire surface becomes gray after sporulation, black in the core and pale gray on the periphery after two

weeks. Old cultures often contain septate, branched, tough, inflated mycelium with melanized hyphae and brownish exudates. Conidiophores are nodulisporium-like branching structures with hyaline, mononematous synonymous, short, minute, dichotomous or trichotomous conidia-forming structures, 1-3 conidia-forming cells arise from each end. In India, Chutulo & Chalannavar (2020) isolated and morphologically identified *Daldinia eschscholtzii* as an endophytic fungus from *Psidium guajava*. *D. eschscholtzii* showed moderately rapid growth on PDA medium. Initially, the fungus forms whitish colonies, which later turn greenish to greyish. Sutthino *et al*. (2021) performed a study to isolate and identify fungal endophytes from three orchids, among which *D. eschscholtzii* was identified based on its morphological characteristics. The fungus produced dark brown color colonies on PDA medium.

Molecular identification: The phylogeny of the fungal endophyte was inferred based on the ITS rDNA sequence. The produced sequence of the ITS region of the organism was approximately 576 bp in length. After the necessary trimming of both ends of the sequence, the data was sent to the NCBI gene bank and given an accession ID: MH368106.1. The examined endophyte-related sequences from the NCBI database were aligned using a BLAST search and MEGA-X was used to construct a phylogenetic tree. In the phylogenetic analysis, the sequence alignment included 42 taxa of representative Hypoxylaceae strains (Fig. 2), including our taxa-*D. eschscholtzii* (MH368106.1). In the BLAST search, the fungal endophyte *Daldinia eschscholtzii* showed 99% homology with previously deposited ITS sequences (*Daldinia eschscholtzii* KX621971.1; *Daldinia eschscholtzii* KY792620.1; *Daldinia eschscholtzii* FJ624265.1; *Daldinia eschscholtzii* MH087114.1; *Daldinia eschscholtzii* MH087106.1) in the gene bank. Maximum Likelihood Tree (ML), Neibor Joining Tree (NJ) and Maximum Parsimony Tree (MP) showed a similar topology. But here only the ML tree was presented. Our isolate MH368106.1 was significantly grouped with *D. eschscholtzii*, confirming the identity of the fungus.

Because of its higher degree of variation, the ITS region of nuclear rDNA can be used to analyze species-level relationships in fungi. The first significant addition to the *Daldinia* taxonomy that included molecular data was made by Johannesson *et al*. (2000) who surveyed the genus across northern Europe and eastern Russia and described a preliminary phylogeny based on ITS sequencing of rDNA for a number of well-characterized specimens. Later, ITS sequence analysis was used extensively to identify *D. eschscholtzii* (Tarman *et al*., 2012; Yuyama *et al*., 2013; Hu *et al*., 2014; Chan *et al*., 2015). Chutulo & Chalannavar (2020) used molecular techniques based on ITS rDNA sequences to identify the endophyte fungus *D. eschscholtzii* from *Psidium guajava*. To construct the pedigree, MEGA-X was used to align multiple sequences from the BLAST search associated with the endophytic isolate. A 99.65% similarity to *D. eschscholtzii* was found in BLAST analysis of the endophytic isolate entered into the GenBank database. Samarakoon *et al*. (2019) reported the identification of *D. eschscholtzii* as an endophyte in *Musa* sp. leaves through molecular features using the ITS sequence. The isolates (MFLUCC19-0154, MFLUCC18-0177, and MFLUCC19-0153) that were the subject of the study formed a large bootstrap supportive cluster with *D. eschscholtzii* (ML=100%, PP=1.00).

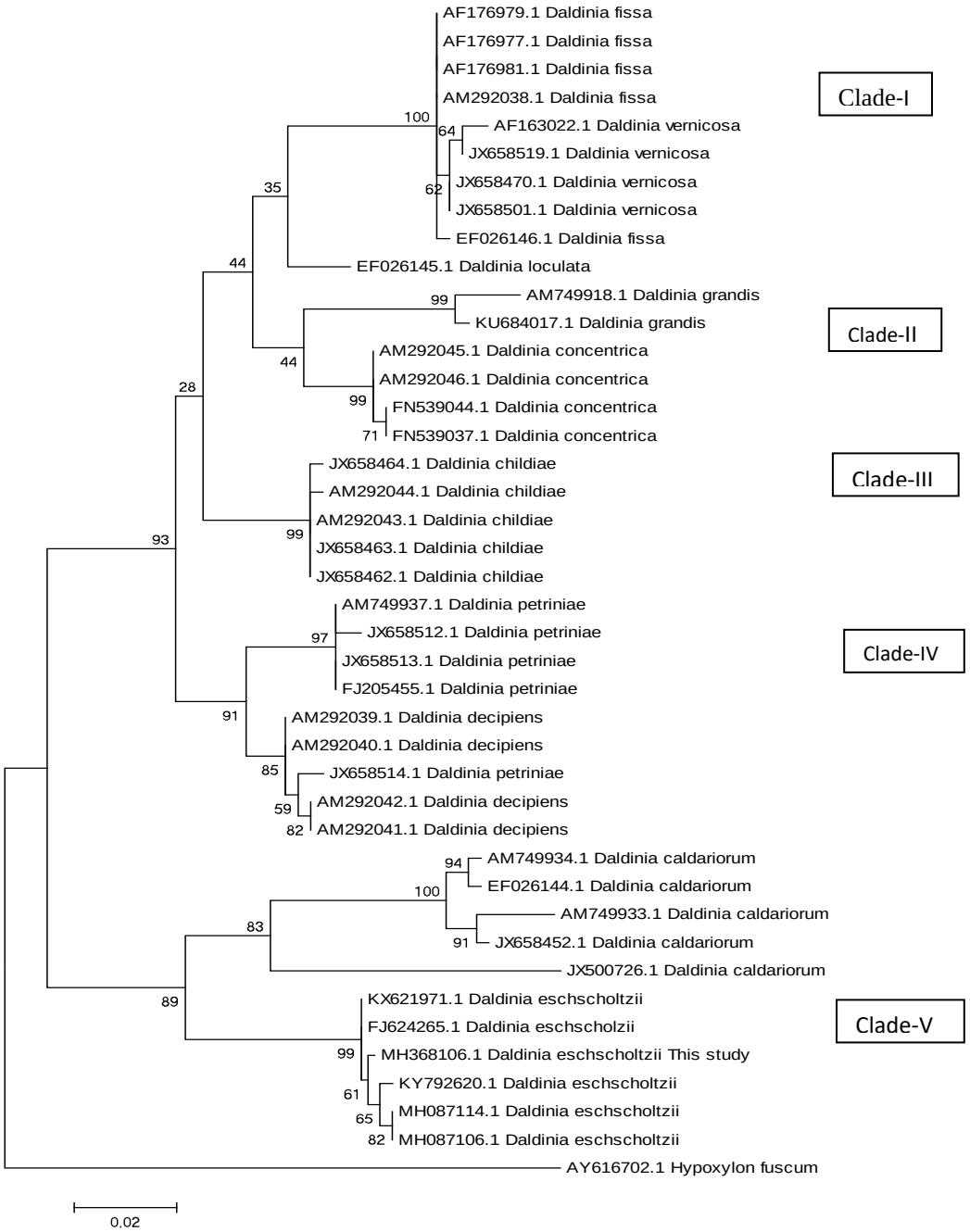

Fig. 2. Maximum likelihood tree generated using the bootstrap value (bootstrap replication=1000) and analysis of the ITS sequence dataset of the examined organism. NCBI accession numbers mentioned before the generic names. Our organism (MH368100.1) is marked with this study

Effect of culture media on mycelial growth of the endophyte: Culture media is the main factor to consider when observing the growth habits of fungi. In this research, significant statistical differences between the culture media were found to support the growth of the fungus tested (Figs. 3A and 3B). The maximum mycelial growth (64.33 mm) was found on PSA medium, followed by 53 mm on PDA, 51.83 mm on CaA medium. The lowest mycelial growth (21.16 mm) was found on RiA medium. The colony appeared a whitish color that became smoky, downy, azonate, or zonate with age, and the reverse side of the plates became blackish in color. The growth rate of the fungus was slower on all media tested. Other environmental factors may be involved in their growth, development and sporulation. Our current results are supported by the results of Ng *et al*. (2016), who mentioned that the colony of *D. eschscholtzii* was initially pale in hue, matured to smoky gray with a slight olive tinge, was fluffy and appeared black on the back, reached 90 mm in 5 days on Sabouraud dextrose agar and V8 Juice Agar, 5-7 days on PDA at 30. Stadler *et al*. (2014) reported that colonies of *D. eschscholtzii* on oat agar reached 90 mm in 5–8 days; Due to the synthesis of pigments tentatively identified as hypoxylxylerone derivatives, the colony first develops as a whitish, tomentose azonate with diffuse margins, then becomes smoky gray with a faint olive tint, and finally melanized. Yuyama *et al*. (2013) evaluated the effect of four culture media, namely potato dextrose, malt extract peptone agar (MEPA), malt extract peptone and minimal medium on vegetative growth and found the best radial growth and mycelial mass on MEPA medium.

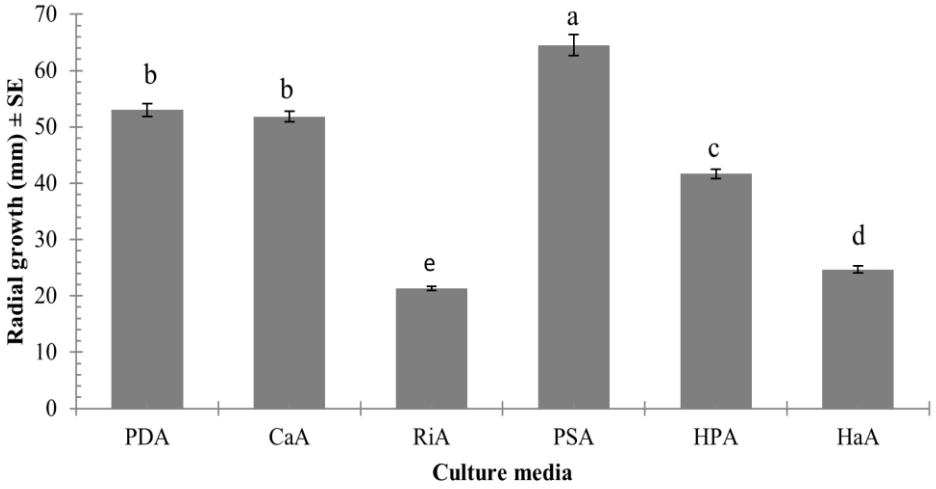

Fig. 3A. The effect of fungal culture media on mycelial growth (mm) of *D. eschscholtzii* at 7 dpi. At the 5% level of significance, the data represent the mean standard error (SE) of six replicates

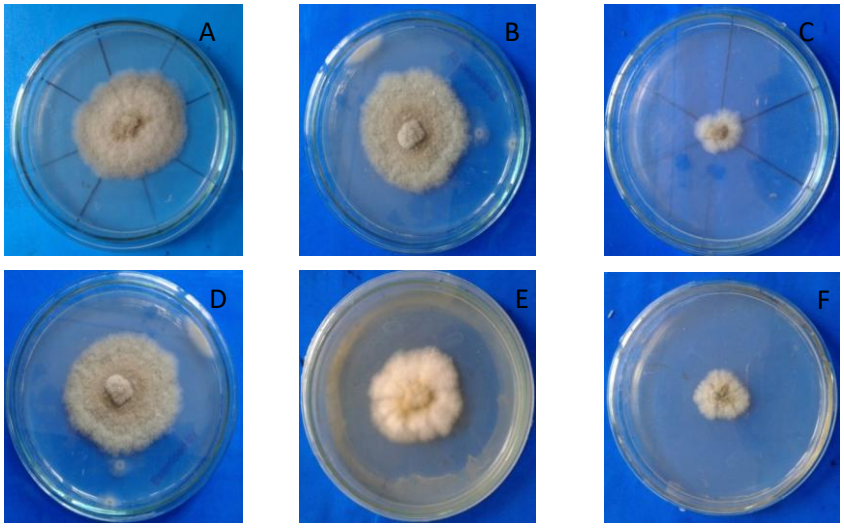

Fig. 3B. The effect of fungal culture media on mycelial growth (mm) of *D. eschscholtzii* at 7 dpi (A: PDA, B: CaA, C: RiA, D: PSA, E: HPA and F: HaA)

Effect of temperature on mycelial growth of the endophyte: Since temperature has a regulating influence on the growth and development of mushrooms, there is always a temperature at which fungi grow optimally. Therefore, the present study investigated how temperature affects the radial mycelial growth of the fungal isolate on PDA media under *in vitro* culture conditions. *D. eschscholtzii* showed the maximum (90 mm) vegetative growth at 25-35°C, whereas the minimum mycelial growth (33.67 mm) was observed at the lowest temperature (15°C) (Figures 4A & 4B). Yuyama *et al*. (2013) studied the vegetative growth of *D. eschscholtzii* isolate around 20-40°C, showing better radial growth at 35°C on malt extract peptone agar (MEPA medium). However, the largest mycelial mass was recorded at 25 and 30°C on MEPA medium. In malt extract peptone (MEP) medium, the maximum biomass results were obtained at 25 and 30°C. Based on all three reviews, a temperature of 30°C was suggested as the ideal temperature for mycelium growth. On PDA medium, *Daldinia caldariorum* showed comparable results (Ng *et al*., 2010). Some *D. caldariorum* and *D. eschscholtzii* cultures develop even faster at 27°C, with radial growth rates exceeding 10 mm/day (Stadler *et al*., 2014). Boddy *et al*. (1985) observed the best mycelial growth of In *D. concentrica* at 25 to 30°C, with growth stopping at 35°C. Yuyama *et al*. (2013) mentioned that the *D. eschscholtzii* isolate grew well up to 40°C. Our result suggests that the fungal endophyte prefers a broad temperature range for growth and development, ranging from 25°C to 35°C.

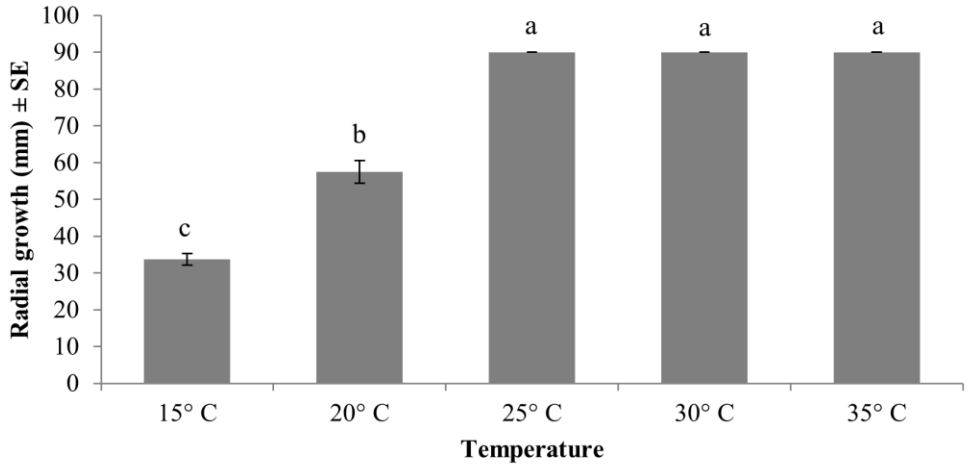

Fig. 4A. The effect of temperature on mycelial growth (mm) of *D. eschscholtzii* at 7 dpi. Value denotes the mean±standard error (SE) of six replications

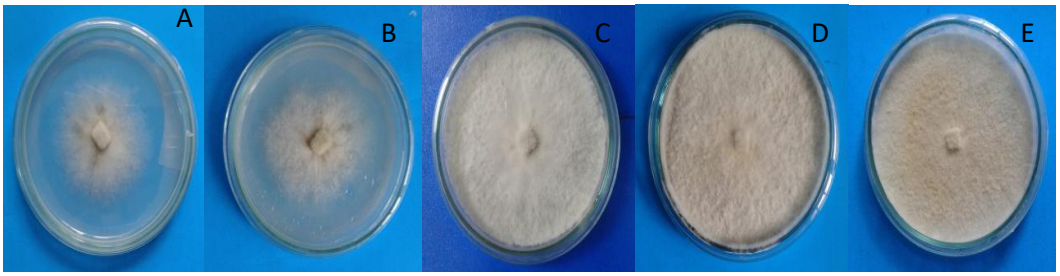

Fig. 4B. The effect of temperature on mycelial growth of *D. eschscholtzii* at 7 dpi (A-15°C; B-20°C; C-25°C; D-30°C and E-35°C)

Since *D. eschscholtzii* is insensitive to pH conditions (data not presented), the influence of pH on growth and development of the fungal endophyte has not received enough attention in the literature.

Conclusion: The endophytic fungus *D. eschscholtzii* has been found to be associated with the fresh leaves of the *Aloe vera* plant. Morphological and molecular features confirmed the identity of the endophyte. It showed the highest radial vegetative growth on PSA medium and favored a broad temperature range of 25-35°C, but incidentally the fungus was found to be insensitive to a pH range, i.e., 5-9. However, the authors firmly believe that further studies may unveil some mysterious information about the association of the endophytic fungus with the *Aloe vera* plant.

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