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Identification and *in vitro* Growth Characteristics of Entomopathogenic fungus-*Aschersonia* sp. in Bangladesh

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Abstract Entomopathogenic fungus-*Aschersonia* sp. was found in the potted plants in the experimental sites of the Department of Zoology, Jahangirnagar University, Bangladesh. Using tissue planting technique, fungus colony was recovered on PDA. Morphology characterization based on mycelium, conidia, colony features as well as sequencing of rDNA of isolated fungus resulted as *Aschersonia* sp. Vegetative growth of the fungus on different fungal culture media, temperature, pH, and the light were conducted. The optimum temperature, pH and light regimes for mycelial growth were recorded at 20°C, pH 7, alternate light and dark condition. The suitable mycelial growth of the fungus was obtained on potato sucrose agar, Richard agar, carrot agar media over ten different culture media studied. To the best of our knowledge, the occurrence of *Aschersonia* sp. in Bangladesh is a new record.

Keywords Entomopathogenic Fungi, Fungal Biology, Molecular, Bangladesh

1. Introduction

The use of entomopathogenic microorganisms to control of insect population is an eco-friendly approach to minimize harmful insecticides use as entomopathogenic fungal spores have found as long lasting, effective alternatives to agrochemicals [1]. The utilization of the insect pathogenic fungal genus *Aschersonia* for control of pest has studied extensively in different countries [2-4]. *Aschersonia aleyrodis* was the first fungal species applied to control of insect in North America with the potential control of citrus whiteflies in Florida (USA) being attained in the early 1900s [5-6]. *Aschersonia placenta* is known to distribute in China, Ghana, India, Malaysia, Thailand, Indonesia, Cameroon, New Guinea, the Philippines, and Vietnam [6]. *Aschersonia* spp. are not hazardous to mammals and some species can be effective bio-control agents against insect [1]. *Aschersonia* Mont. belongs to the Ascomycota, Pezizomycotina, Sordariomycetes, Hypocreomycetidae, Hypocreales, and Clavicipitaceae. This fungus has been recognized as a very important biological control agent which able to cause remarkable epizootic disease in whiteflies (Aleyrodidae) and scale insects (Coccidae) in the tropical and subtropical region [7]. Genus *Aschersonia* is consists of 80 described species (Mycobank). Molecular characterization seems to be important for better understanding population structure, ecology, isolate, and usefulness in the selection of an appropriate fungal antagonistic biocontrol agent [8-9]. Random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) markers have applied to investigate relationships among *Aschersonia* species and isolates [10]. In recent years, molecular phylogenetic analyses based on inter transcribed spacer (ITS) of rDNA, translation elongation factor 1-α (TEF1-α), β-tubulin and mitochondrial cytochrome oxidase subunit (COI) were carried out to determine the relationships of new species to other species of *Hypocrella* (Anamorph: *Aschersonia*) [11-12]. Studies on growth characteristics are important for the formulation of entomopathogenic fungi. Vegetative growth and sporulation on artificial culture media are key biological characteristics of entomopathogenic fungi. The basic nutritional requirements could have an extensive effect on culture mycelial growth, sporulation and morphology in entomopathogenic fungi [7]. The present experiment was conducted to identify entomopathogenic fungus using morphological and molecular tools, to study the growth characteristics of it for further formulation of the product.

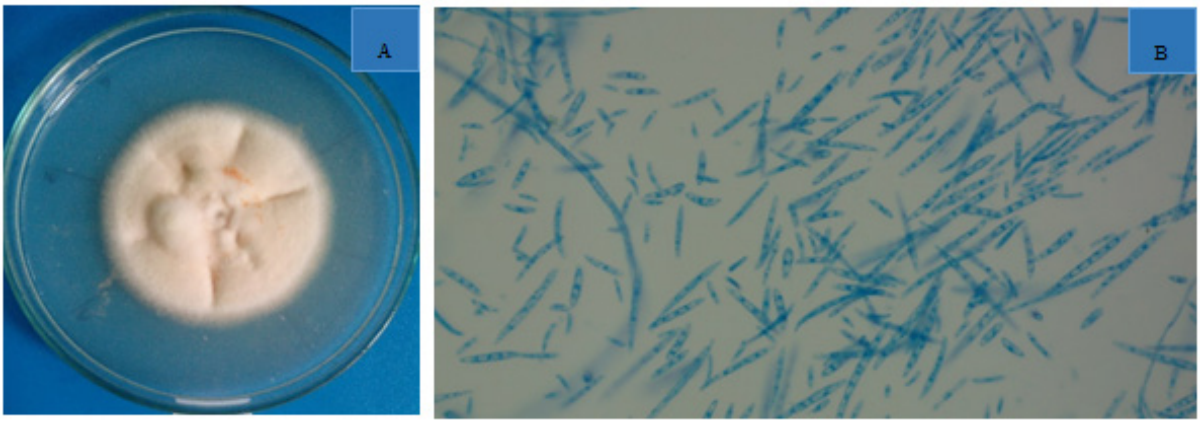

Figure 1. A. The colony of *Aschersonia* sp. on fungal culture media: B. Microscopic view of the spore of *Aschersonia* sp JUEPF-1 (40X×10X magnification)

2. Materials and Methods

2.1. Isolation and Morphological Identification of the Fungus

Aloe vera leaf samples were brought into Mycology and Plant Pathology laboratory from potted plants using sterile polyethylene bag. The leaf samples were chopped into small pieces, after sterilization using NaOCl (0.5%) solution for 5 minutes and were rinsed with distilled water several times. These leaf-cutting samples were placed into PDA (potato dextrose agar) media and were incubated under 12/12 hours dark and light condition at 25±2°C for 3-4 days. Mycelia growth of growing fungus colony was transferred to fresh PDA plates as well as PDA slants to obtain a pure culture. The pure culture of the isolated fungus was identified microscopically (Figure 1) and strain name was given to the fungus. Generally, the fungal colony on cultural media was light brown in color, produced creamy orange spore mass and conidial shape was fusoid, pointed towards both ends. These features were used to identify this fungus as described by Liu [6] and Wang [13].

2.2. Molecular Characterization

The fungal pathogen was molecularly characterized with the help of commercial service provided by Invent Technology, Dhaka, Bangladesh. Fungus genomic DNA samples were extracted using the Maxwell Cell Kit (AS1030, Promega, USA). The primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) were used for the PCR reaction [14]. The PCR reaction was performed with 20 ng of genomic DNA as the template in a 25-µl reaction mixture having a LA Taq (TAKARA BIO INC, Japan). The thermal cycle was performed with activation of Taq polymerase at 94°C for 1 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 was used to purify the amplification products (Promega, USA). The purified PCR products of 558 bp were sequenced by using two primers in First BASE Laboratories SdnBhd (Malaysia).

Sequence alignment and phylogenetic analyses DNA sequences were checked with BioEdit [29] and MEGA6 [15-16]. Sequencing datas were submitted to NCBI, and received an accession number (MH368145.1). A BLAST search with the ITS sequences were used to reveal the closest matching taxa in family-Clavicipitaceae. Multiple sequence alignments were done using MEGA6 [16]. Data was converted from fasta to MEGA format with Clustal W [30]. The models of evolution were determined under the Akaike information criterion (AIC). The model selected was Tamura-3 parameter for analysis. Maximum likelihood (ML), Neighbor-joining (NJ), and Maximum parsimony (MP) analysis were done and robustness of the branches were determined with 1000 bootstrap replicates along with max-trees set at 1000. The number of replications was inferred using the stopping criterion. Bootstrap values greater than 60% were accepted.

2.3. Growth Characteristics of the Fungus

The effects of temperature on mycelial growth of the *Aschersonia sp.* were studied as described by Mello et al. [17]. Different temperatures (15, 20, 25 and 30°C) were maintained for the mycelial growth of the pathogen on PDA in an incubator. The mycelial growth was recorded at 3 and 7 days post inoculation (dpi), respectively. The effect of light on the mycelial growth of the pathogen was done by exposing the inoculated culture to alternate cycles of 24 h light, 24 h dark and 12 h light and 12 h dark in an environment chamber, in which at room temperature (25 ± 2°C) was maintained Mello et al. [17]. The effect of pH on the growth of the fungus was assayed on PDA medium. Different pH levels viz., 6.0, 7.0, 8.0 and 9.0 were used. The pH levels of the medium were adjusted in a digital pH

meter using 0.1 N Hydrochloric acid and 0.1 N Sodium hydroxide Rathore et al. [18]. Ten different culture media (Potato Dextrose Agar (PDA), Yeast Extract Agar (YEA), Honey Peptone Agar (HPA), Malt agar (MA), Hansens's Agar medium (HA), Sabouraud Glucose Agar (SGA), Kauffman's Agar (KA), Potato Sucrose Agar (PSA), Richard's medium (RA), and Carrot Agar (CA) were used to assay the mycelial growth of the pathogen Fovo et al. [19].

3. Results and Discussion

3.1. Morphological and Molecular Characterization

In Figure 1, entomopathogenic fungus-*Aschersonia* sp. JUEPF-1 was identified based on colony morphology, morphological characteristic of conidia as according to Liu [6], and Wang and Song [13]. Percent homology of rDNA sequence of ITS region (MH368145.1) was compared with formerly identified fungi AY225327.1 *Aschersonia* sp., AY214459.1 *Aschersonia* sp., AY225331.1 *Aschersonia* sp., AY225330.1 *Aschersonia* sp., and AY225332.1 *Aschersonia* sp. (Figure 2). Thirty-one fungal taxa were selected from the NCBI database for phylogenetic analysis. In maximum parsimony tree (Figure 2), there are five different clades were found in the phylogenetic tree. Clade-I consists of six taxa of *Beauveria bassiana* and *Cordyceps militaris* with 98% bootstrap value. Clade-II consists of six taxa under the genus *Aschersonia minutispora, A. narathiwatensis*, *Hypocrella calendulina* with 100% bootstrap value. Clade-III consists of two sister clades with one was included *Fusarium lateritium* and other one had *Aschersonia* with 99% bootstrap value. Clade-IV was strongly supported (100% bootstrap value) the homology among *Aschersonia* in which our fungus is located. Clade-V had three taxa of *Metarhizium anisopliae* with 100% bootstrap value and these organisms were formed a separate clade of the phylogenetic tree. Based on molecular evidence, it is clearly indicated that our studied fungus is *Aschersonia* sp, and it seems that entomopathogenic fungi-*Aschersonia* sp. is polymorphic.

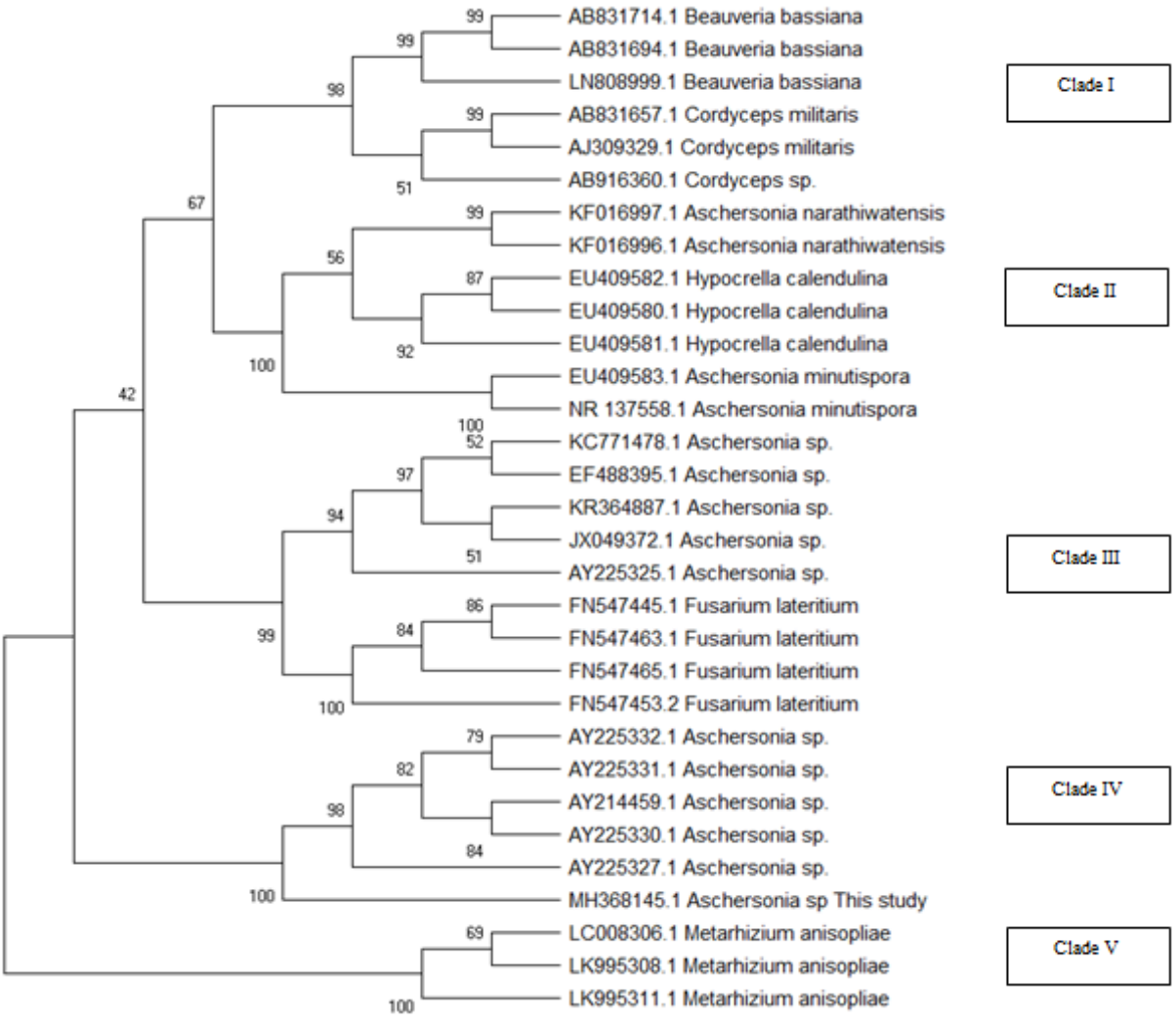

Figure 2. Maximum parsimony tree of the ITS region of rDNA sequence of the studied organism with bootstrap value (Replication=1000)

3.2. Growth Characteristics

It is universal that fungi are capable of indefinite growth at its favorable conditions where physiological and environmental factors play an important role.

3.2.1. Effect of Culture Media

In the present study, the maximum mycelial growth (21.22 mm) of *Aschersonia sp.* was obtained on SGA culture media, which was statistically identical to Carrot agar (CA) media but significantly different from others culture media at 3 days post incubation (dpi) (Figure 3 and 4). There was no mycelial growth on Malt extract agar media. But at 7 dpi, the highest radial mycelial growth (47.56 mm) was obtained on Carrot agar (CA) media, which statistically identical to potato sucrose agar (47.33 mm) and Richard's medium (47.00 mm); the least mycelial growth (8.33 mm) was recorded on MA media (Figure 3 and 4). The most extensively used potato dextrose agar (PDA) media exhibited 37.22 mm radial growth of the studied fungus, which was much lower compared to other culture media used in the present study. Present findings supported by Zhengyi [20] who obtained quicker growth and more sporulation of *Aschersonia aleryoidis* on PSA media compared to other media tested. Our results are in contradictory with the findings of Sun et al. [21] who mentioned that the mycelium of *Aschersonia aleyrodis* able to grow better on PDA and corn flour sucrose yeast extract agar medium than others. Moreover, the fungus produced more spores on PDA, PSA, and corn flour sucrose yeast extract agar media. In another study, it reported that the growth and sporulation seemed to be much higher in semisolid than in liquid media. Amongst several plant media verified, pumpkin consistently gave the maximum mycelial growth and sporulation of *Aschersonia placenta* [22]. In an earlier study, seven synthetic fungal culture media were studied, *Aschersonia placenta* grew best on Sabouraud dextrose agar with yeast extract (SDAY) and Sabouraud dextrose agar (SDA) with pasteurized milk (SDA+M) based on the colony radius measurement; however, based on the ability of conidia formation, PDA and SDAY were found as the most suitable media [23]. In our study, ten different culture media tested, which not studied by earlier workers, even we could not detect the fungus in species level, and presently studied fungus might be different species.

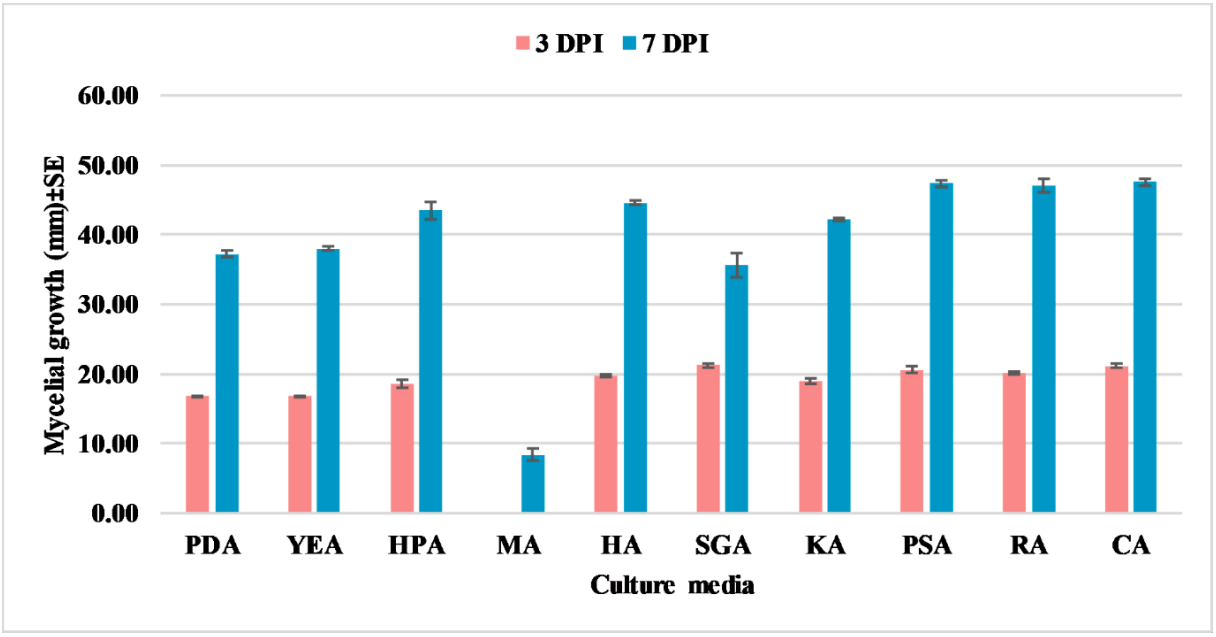

Figure 3. Effect of different fungal culture media on mycelial growth of *Aschersonia* sp. at 3 and 7 days post incubation (dpi). PDA (Potato Dextrose Agar, YEA (Yeast Extract Agar), HPA (Honey Peptone Agar), MA (Malt agar), HA (Hansens's Agar medium), SGA (Sabouraud Glucose Agar), KA (Kauffman's Agar), PSA (Potato Sucrose Agar), RA (Richard's medium), and CA (Carrot Agar). Data represent as mean±Standard error of six replications

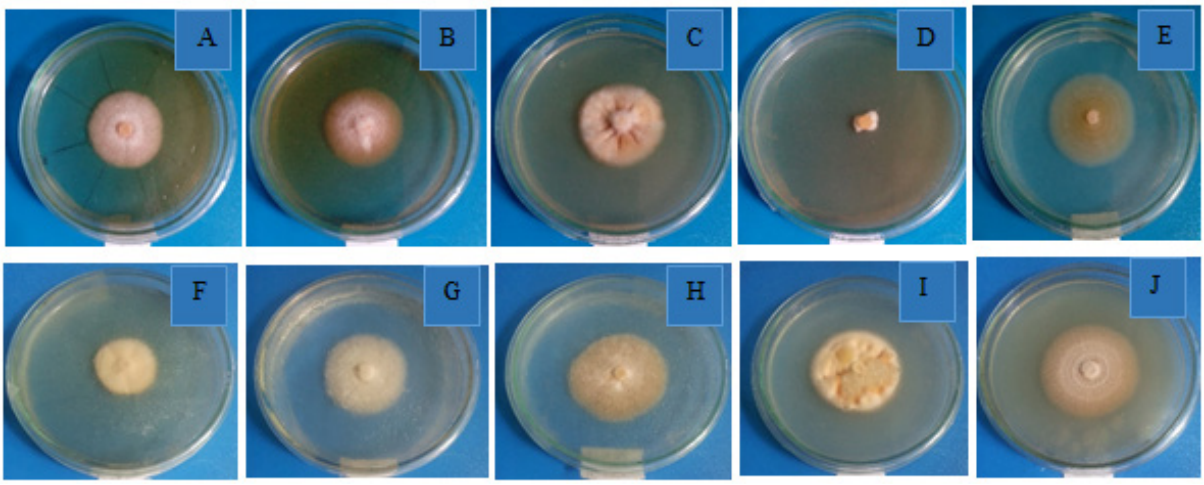

Figure 4. Effect of different fungal culture media on mycelial growth of *Aschersonia* sp. at 7 days post incubation (dpi). A: PDA (Potato Dextrose Agar, B: YEA (Yeast Extract Agar), C: HPA (Honey Peptone Agar), D: MA (Malt agar), E: HA (Hensens's Agar medium), F: SGA (Sabouraud Glucose Agar), G: KA (Kauffman's Agar), H: PSA (Potato Sucrose Agar), I: RA (Richard's medium), and J: CA (Carrot Agar)

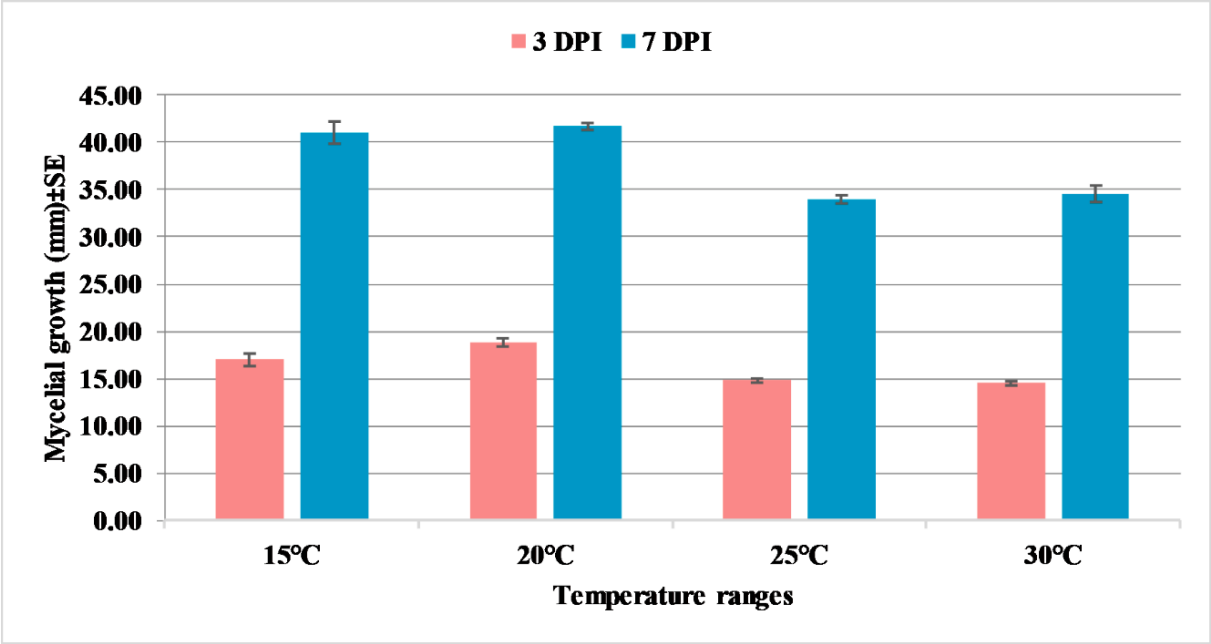

Figure 5. Effect of temperature ranges on mycelial growth of *Aschersonia sp.* at 3 and 7 days post inoculation (dpi). Data represent as mean±Standard error of six replications

3.2.2. Effect of Temperature

Temperature is one of the important abiotic factors affecting development and sporulation of entomopathogenic fungi. In the current study, at 3dpi, the radial mycelial growth of *Aschersonia sp.* was maximum (18.80 mm) at 20°C, followed by a temperature at 15°C (17.0 mm) and the minimum (14.50 mm) was found at the higher temperature 30°C (Figure 5). There were similar trends of mycelial growth of the studied fungus was found at 7 dpi. The highest radial growth (41.70 mm) obtained at 20°C, which is identical to 15°C and least was 34.00 mm at 25°C, which was close to 30°C (Figure 5). Present results are partially conformity with the findings of Sun et al. [21] who obtained the mycelial growth of *Aschersonia aleyrodis* over 10 to 30°C and the optimum temperature was 25°C. Zhengyi [20] reported that *Aschersonia aleryoidis* was able to grow and sporulate from 10°C to 32°C with 20°C-30°C being the optimum temperature. Conidial germination of this fungus ranged from 5°C to 30°C temperature, with 20°C to 30°C being the optimum. However, Homrahud et al. [23] indicated the slow growth rate and few conidia of *Aschersonia placenta* isolate Asp001at 25°C. The optimum conidial germination and growth of the germ tube of *Aschersonia placenta* found over a temperature range of 25–30°C. Similarly, Ibrahim et al. [22] cited that a temperature of 30°C resulted in the longest germ tube formation over other temperatures tested.

These previous studies indicated that entomopathogenic fungus able to adapt fairly over broad temperature ranges depending on the prevalence of the environmental condition.

3.2.3. Effect of pH

pH is one of the key abiotic factors, which influences not only the survival of the entomopathogenic fungi in the field condition, but also their virulence against the target insect population [25-28]. In the present study, there was statistically significant difference observed among the ranges of pH at both 3 dpi and 7 dpi (Figure 6). At 3 dpi, the highest mycelial growth (19.56 mm) of *Aschersonia sp.* recorded at pH 7, the growth rate was statistically identical to pH 6. The lowest mycelial growth (17.44 mm) was found at pH 9. Similarly, at 7 dpi, the maximum mycelial growth (44.11 mm) observed at pH 7, the growth rate was significantly different to pH 6, pH 8 and pH 9. The present result indicated pH 7 is the optimum for mycelial growth of *Aschersonia* sp. that is in agreement with findings of previous workers. Zhengyi [20] cited that the pH range for the vegetative growth and sporulation of *Aschersonia aleryoidis* was found the optimum with ´pH 6-7. However, Ibrahim et al. [22] reported that the pH range of 5.0–6.0 of *Aschersonia placenta* found optimum for vegetative growth and development. Hallsworth and Magan [25] observed that the mycelial growth of entomopathogenic fungi such as *Beauveria bassiana, Paecillomyces farinosus*, and *Metarhizium anisopliae* was found suitable at a pH range of 5 to 8. They mentioned that the entomopathogenic fungal species (EPFS) could grow over a broad range of pH in contrast to some other fungi. The entomopathogenic fungal species have the ability to regulate their cytosolic pH better than the other species, which have optimal growth only at a narrow pH range. Our present findings are in conformity with the result of Hallsworth and Magan [25] who found EPFS grew over a wide pH range. St Leger et al. [26] studied the effect of pH on the expression of various cuticle-degrading enzymes secreted by *Metarhizium anisopliae*. They found that the genes encoding various cuticle-degrading enzymes expressed at the pH optimal for the specific enzyme. They also observed that the alkaline pH of the insect cuticle generally triggers the secretion of protein-degrading enzymes like proteases etc. as a result fungus can penetrate through the degrading the hard surface of the cuticle.

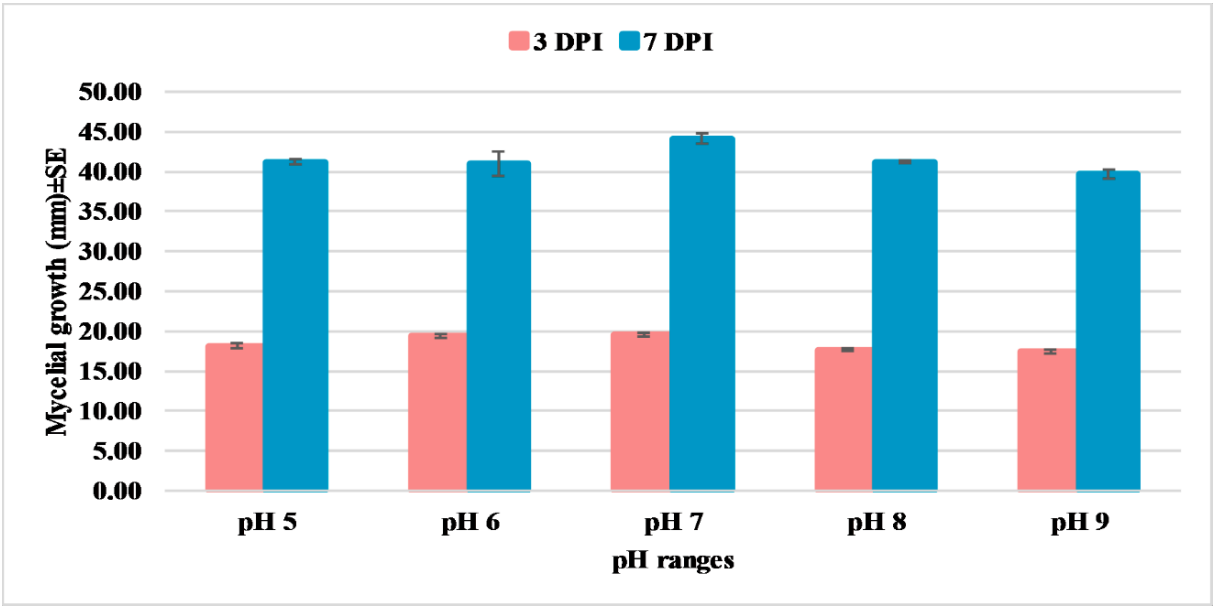

Figure 6. Effect of pH on mycelial growth of *Aschersonia* sp. at 3 and 7 days post inoculation (dpi). Data represent as mean±Standard error of six replications

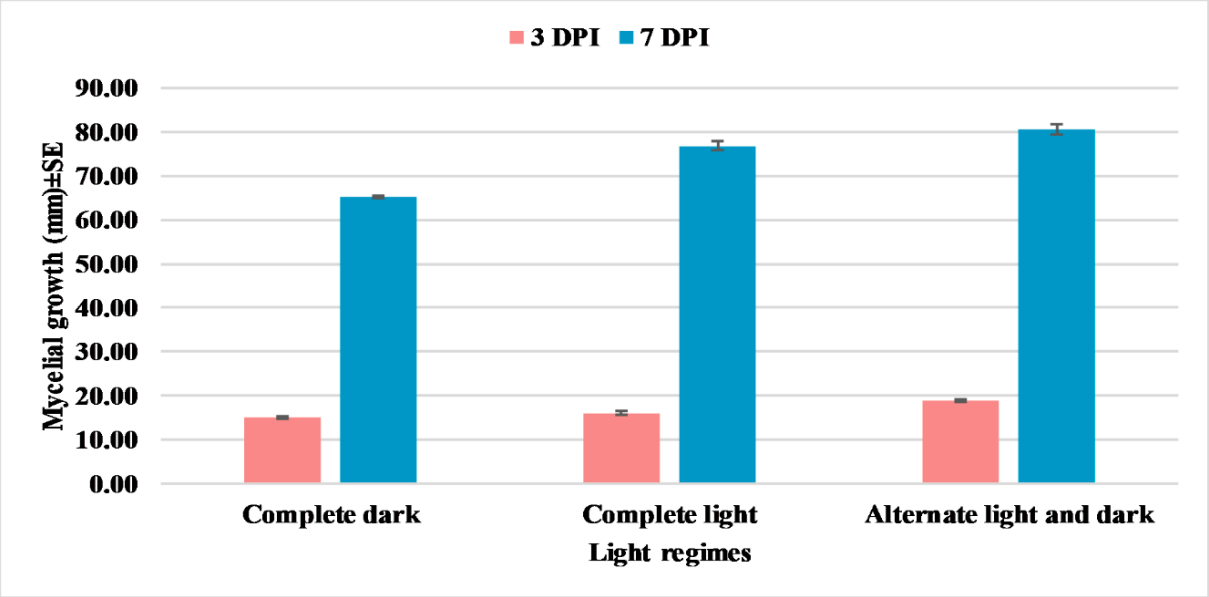

Figure 7. Effect of light regimes on mycelial growth of *Aschersonia* sp. at 3 dpi and 7 days post-inoculation (dpi). Data represent as mean±Standard error of six replications

3.2.4. Effect of Light

In the present study, *Aschersonia sp.* grew best (18.89 mm) on Richard's medium under alternate light and dark condition which was significantly different from complete light and complete dark condition at 3 dpi (Figure 7). There were similar trends of vegetative growth of the studied fungus was observed at 7 dpi, in which the highest mean radial mycelial growth (80.56 mm) was obtained due to alternate light and dark condition, followed by complete light (76.78 mm) and least due to complete darkness (65.22 mm). However, Sun et al. [21] mentioned that illumination had no remarkable effect on the growth of the *Aschersonia aleyrodis*, but 12-hour intermittent illumination was in favors of the spore-bearing. Alves et al. [24] concluded that amongst the 12 combinations of temperature and photoperiod tested, a photoperiod of 16 hours among four different light regimes was found most suitable for development and sporulation of *Metarhizium anisopliae* isolate SPL - 3B. In our study, the change of mycelial color was evident due to exposure of light, the colony was a bit whitish under complete dark condition whereas 24-hours light provided light brown colony and the concentric ring was found in case 12h/12 light and dark condition. This change of color indicated that light plays an important role in the growth and development of *Aschersonia* sp.

4. Conclusions

Chemical composition of the fungal culture media and environmental factors affect the mycelial growth of *Aschersonia* sp differently. Appropriate abiotic factors should take into considered for infection assay (both *in vitro* and *in vivo*) and future formulation of the studied fungus as a biocontrol agent.

Acknowledgments

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