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First Report of *Pseudopestalotiopsis theae* Causing *Aloe vera* Leaf Spot Disease in Bangladesh

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Abstract

Aloe vera (L.) Burm.f. is one of the important medicinal plants, has been commercially cultivated in the Northern part of Bangladesh. An experiment was conducted to detect *A. vera* leaf disease collected from Natore district, Bangladesh. Fungal leaf spot disease caused by *Pseudopestalotiopsis theae* (Sawada) Maharachch., K.D. Hyde & Crous was identified through morphological features and sequencing of internal transcribed spacer region of ribosomal DNA. After submitting nucleotide sequences to NCBI, we received an accession number MH333081.1: *Pseudopestalotiopsis theae*. The growth pattern of the isolated fungal pathogen was evaluated on different solid culture media, at different temperature and light conditions. The results showed the maximum mycelial growth of the fungus on the Richard Agar medium under the complete dark condition at 25˚C. We evaluated fungal antagonists against the isolated pathogenic fungus, in which *Trichoderma asperellum* showed optimistic results. Synthetic fungicides—Tilt 250EC and Ridomil Gold completely inhibited the studied fungus's vegetative growth. *Pseudopestalotiopsis theae* causing *A. vera* leaf spot disease in Bangladesh is a new record to the best of our knowledge.

Keywords

Fungal Biology, Molecular, Growth Characters, Bio-Control Agents, Fungicides

1. Introduction

The cultivation of medicinal plants has become one of the vital earning sources

for rural people in Bangladesh. *Aloe vera* plants are being cultivated in the northern parts of Bangladesh. These plants are used in medicine preparation, cosmetics industries and juice [1]. The contamination in leaves with fungi and other microbes is of public importance. Besides, fungal pathogens produce mycotoxins, which is a human health concern. Due to the succulent nature of the *A. vera* plants, they are susceptible to various pathogenic microorganisms responsible for qualitative and quantitative loss and incur the lower production of the plant. Some fungal diseases of *Aloe* have been reported worldwide and in Bangladesh. For instance, leaf spot disease caused by *Alternaria alternata* [2]; *Nigrospora oryzae* [3]; *Fusarium oxysporum* [4]; *Colletotrichum siamense* [5]. Generally, mycologists use classical taxonomy to identify fungi. However, diagnosing the fungi at species rank accurately is challenging due to fewer distinct taxonomic features. Now, mycologists use molecular data to identify fungi using sequencing of nuclear ribosomal DNA, translation elongation factor, beta-tubulin gene, etc. [6]. Three species—*Pseudopestalotiopsis camelliae-sinensis*, *Neopestalotiopsis clavispora*, and *Pestalotiopsis camelliae* causing grey blight disease of tea in China was described using molecular techniques as mentioned above. The ITS is the most useful marker for detecting fungi at species rank due to the fastest evolving portion of the rRNA cistron. Besides, internal transcribed spacer region has been chosen as the official barcode to identify fungi due to their difference between intraspecific and interspecific variation [7].

The culture media plays a vital role in the growth, development and sporulation of fungi. Besides, relevant environmental factors such as temperature, light, etc. are essential for the germination of fungal spores, the development of germ tubes, thereby disease development. Farmers generally use fungicides to manage fungal diseases. However, harmful chemical fungicides are detrimental to the environment, animal and human health. The use of biocontrol agents could be an effective alternative to chemical pesticides. Therefore, an experiment was conducted to detect the causal agent of *A. vera* leaf spot disease; to study the influence of fungal culture media, temperature, light on the isolated fungus; to evaluate the efficacy of selected bio-control fungi and chemical fungicides against the isolated fungus.

2. Materials and Methods

2.1. Isolation and Identification of the Fungus

Diseased *A. vera* leaf samples with diagnostic symptoms were collected from the Northern part (Natore district) of Bangladesh and the Botanical Garden, Jahangirnagar University, Bangladesh. Tissue planting method was employed to isolate fungus and was identified based on colony morphology and morphological features of mycelia and conidia [8]. The pathogenicity test of the isolated fungus was carried out following the modified "detached leaf technique" [9].

Two universal primers ITS4 (5-TCCTCCGCTTATTGATATGC-3) and ITS5 (5-GGAAGTAAAAGTCGTAACAAGG-3) were employed to amplify the fun-

gus's ITS region. [10]. The PCR reaction was performed according to Sikder *et al.* [11]. The purified PCR products were sequenced in the Sanger sequencing platform, First BASE Laboratories, Sdn Bhd, Malaysia. Sequencing data were submitted to NCBI and received an accession number. Sequence data were compared with other nucleotide sequences after retrieving from the NCBI GenBank database. The maximum likelihood tree was generated using MEGA 6 software.

2.2. Effect of Culture Media, Temperature and Light on the Vegetative Growth of the Fungus

To assess the fungal mycelial growth pattern, six discrete fungal culture media, namely Potato Dextrose Agar (PDA), Potato Sucrose Agar (PSA), Carrot Agar (CA), Richard Agar (RA), Honey Peptone Agar (HPA), Honey Agar (HA) were used [12]. The five different temperatures conditions were tested to evaluate the mycelial growth of the fungus was according to Sultana *et al.* [13]. The influence of three photoperiodic conditions on the vegetative growth of the fungus was also investigated [14]. Data were recorded at 7 days post-incubation (dpi).

2.3. *In Vitro* Mycelial Growth Inhibition of the Fungal Pathogen

The vegetative growth inhibition of the target fungus was assessed using the dual culture technique, in which three biological control agents namely—*Trichoderma reesei*, *Trichoderma harzianum*, and *Trichoderma asperellum* were used [15]. The efficacy of three different synthetic fungicides Tilt 250EC @ 100 ppm, 250 ppm and 500 ppm; and Ridomil gold MZ 68 WP and Amistar Top 325 SC @ 250 ppm, 500 ppm, and 750 ppm were evaluated on the isolated fungus by poison food techniques under laboratory conditions [16]. The PDA plate containing the tested fungus without any bio-control agent or fungicide was served as control. The percent vegetative growth inhibition of the fungus was estimated at 7 dpi [17].

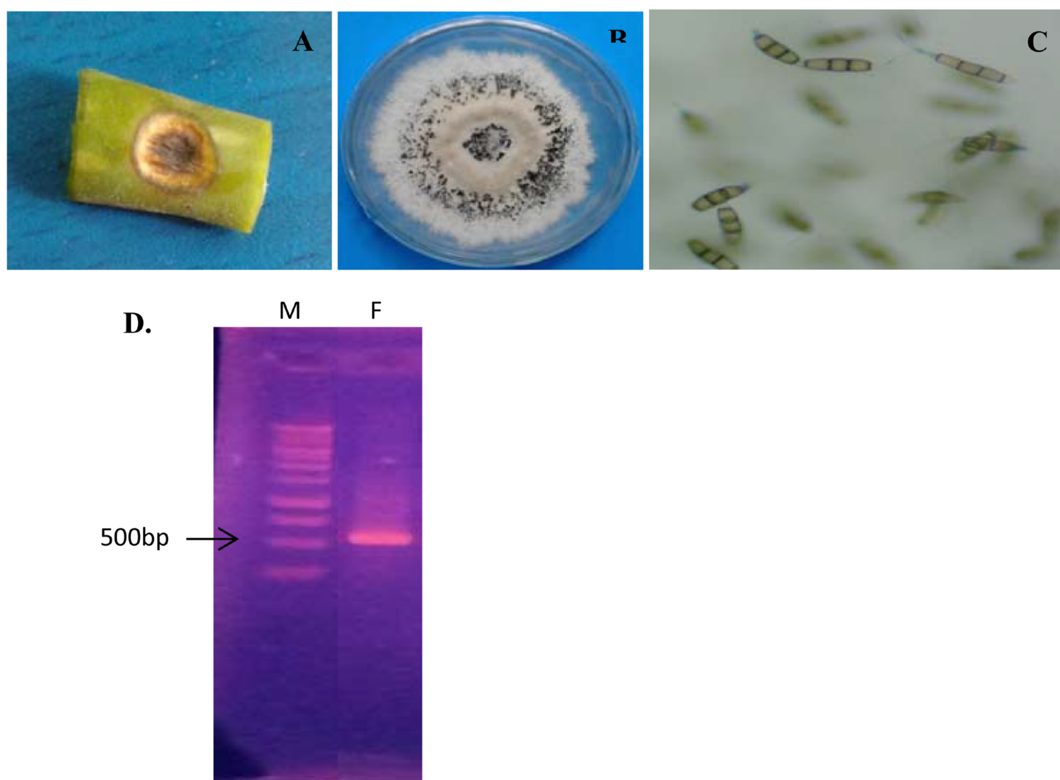
2.4. Statistical Analysis

Data on the effects of fungal culture media, temperature, light, bio-control agents, and chemical fungicides on the mycelial growth of the fungus were checked for normality and homogeneity of variance. Data were analyzed using one-way ANOVA with Duncan's Post-Hoc test in SPSS-16.

3. Results and Discussion

3.1. Isolation and Identification of the Fungus

Pseudopestalotiopsis theae (*P. theae*) formed a rounded spot with black color in the center and a brown margin on the plant's upper leaves (**Figure 1(A)**). The fungus produced white color colony with an irregular margin and formed sclerotia on the PDA medium (**Figure 1(B)**). Acervuli were scattered, globose to lenticular. Conidia had three median cells with pigmented light brown and dark



M= 10,000 bp ladder; F: *Pseudopestalotiopsis theae*

Figure 1. (A) Symptoms of *P. theae* causing leaf spot disease in the leaf of *Aloe vera* plant; (B) Vegetative growth of the fungus on PDA medium; (C) Microscopic view of conidia (100×); profiles of amplification of rDNA-internal transcribed spacer region of the targeted fungus obtained with ITS4 and ITS5 primers.

brown margin. The apical cell of conidia contains 3 flagella and the basal cell contains single flagella. Conidia are fusiform in shape (**Figure 1(C)**). Similar morphological features were also described by another researcher [8]. The pathogenicity test confirmed the isolated pathogenic fungus—*P. theae* through Koch postulates. The typical dark brown and black irregular spots and lesions were observed on the artificially inoculated leaves after 7 - 10 days of inoculation.

The PCR product of the studied fungus was approximately 650 bp in size (**Figure 1(D)**) which was further confirmed via Sanger sequencing. In BLAST search, our studied organism MH333081.1 *Pseudopestalotiopsis theae* showed 98% sequence similarity with previously identified species, KM111476.1 *Pseudopestalotiopsis theae*; KX757714.1 *Pseudopestalotiopsis camelliae-sinensis*; KX757707.1 *Pseudopestalotiopsis camelliae-sinensis*; HQ832793.1 *Pseudopestalotiopsis theae* and MF495464.1 *Pseudopestalotiopsis theae*. The clustering of the species confirmed our organism as *Pseudopestalotiopsis theae* There were three major clades in the maximum likelihood (ML) tree; the first clade consists of both *Pseudopestalotiopsis camelliae-sinensis* and *Pseudopestalotiopsis theae* i.e., theae group in which our studied organism located with highly similar taxa (**Figure 2**). The second clade had a group of species belonging to the genus-*Neopestalotiopsis*. The third clade had different species of the genus-*Pestalotiopsis* (**Figure 2**).

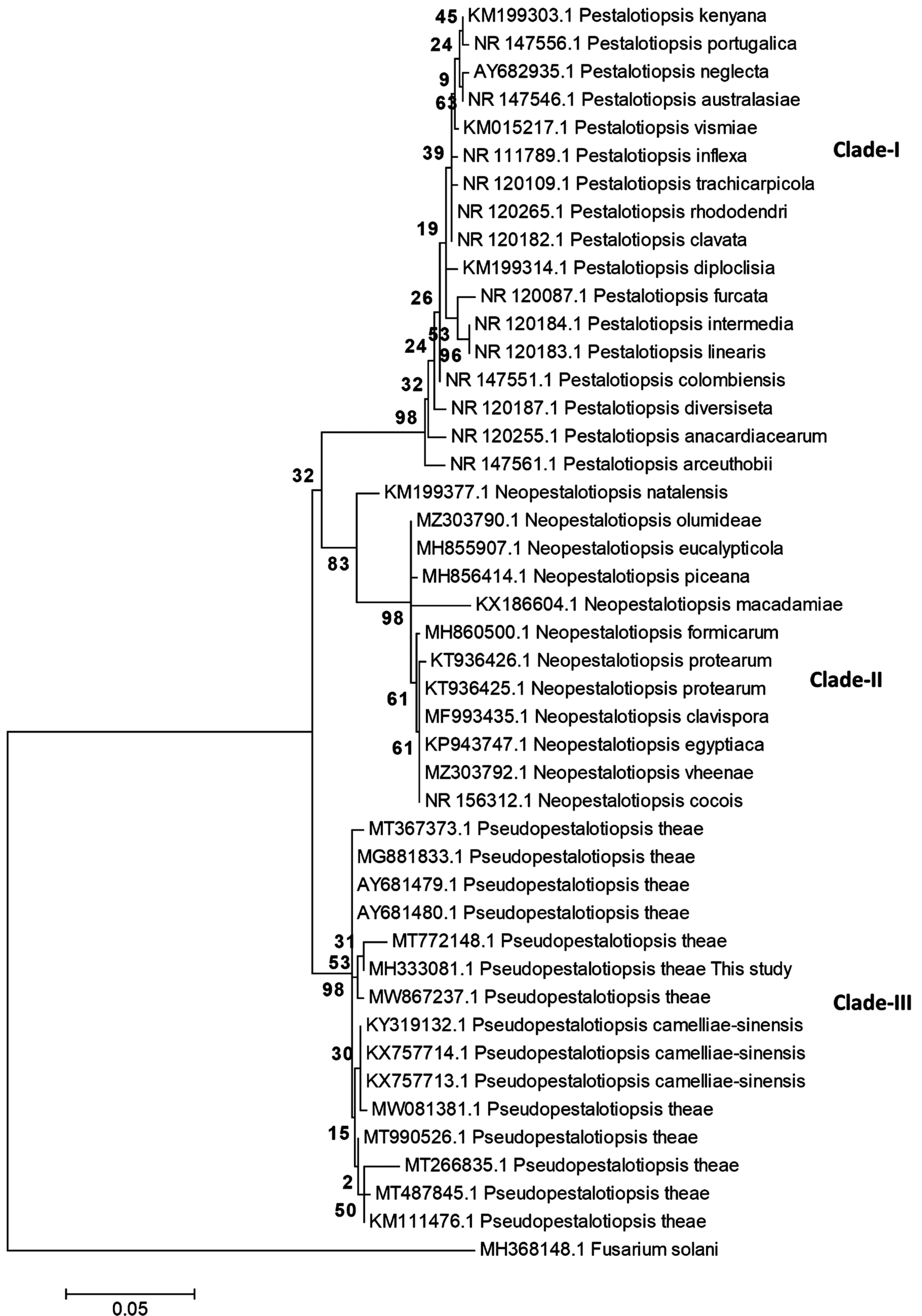


Figure 2. A maximum-likelihood tree of the 18S rDNA sequences of the studied fungal organism with retrieved NCBI sequence data. Our studied organism (MH333081.1) has been marked as "This study".

3.2. Growth Characteristics of the Studied Fungus

The vegetative growth pattern of the fungus on different solid culture media *viz.*, PDA, CA, RA, PSA, HPA, and HA was distinct and there were significant differences among the fungal culture media (**Figure 3(A)**). In our investigation, RA and PSA gave the maximum mycelial growth of *P. theae* compared to commonly used PDA media. Although RA medium supported the highest mycelial development, sclerotium formation was the maximum on PSA medium. Our results agree with the findings of previous worker, who cited the radial mycelial growth rates of *Pestalotiopsis* spp. (*Pestalotiopsis fici*, *P. guepinii* and *P. palmarum*) were affected by culture media [18]. The PSA was the most favorable for fast radial growth of mycelium than Pestacia Leaf Agar and Water Agar media. The V8 juice agar supported the most rapid mycelial growth rate of *Pestalotiopsis microspora* compared to PDA and malt extract agar [19]. In a lab bioassay, *Pestalotiopsis funerea has been* reported the most remarkable growth rate of on TAKAY medium compared to other four different fungal media since TAKAY medium consists of many nutrients and compounds [20].

In our study, an increasing trend of mycelial growth of the fungus was observed up to 25˚C temperature and started to decline afterward (**Figure 3(B)**). Notably, there was no mycelial growth recorded at 35˚C temperature. Current findings conform to the results of other investigators who observed the mycelial growth of three species of *Pestalotiopsis* (*Pestalotiopsis fici*, *Pestalotiopsis guepinii* and *Pestalotiopsis palmarum*) was attained a maximum of 94% of the petri plates after five days of incubation [18] [21]. Likewise, *Pestalotiopsis funerea* grew the maximum at 25˚C [22]. Moreover, the growth rate of *Pestalotiopsis microspora* was found to be increased with temperature and attained their optimum at 23˚C [19]. Furthermore, it was reported that the temperature ranges between 22˚C - 28˚C were optimum for the mycelial growth of all isolates of *P. microspora* [18]. Importantly, *P. theae* did not grow at higher temperatures (35˚C) [23]. Similar results were also reported by other researchers who found that *Pestalotiopsis microspora* failed to grow at 33˚C [19] and *Pestalotiopsis* at 35˚C [23]. The mechanism could be due to the inactivation of key enzymes of the metabolic pathway by elevated temperature [24] [25].

The light was found as an essential determinant for the vegetative growth and development of fungi. The growth rate of *P. theae* was the maximum under complete dark conditions, surprisingly there was no mycelial growth of the fungus found under continuous light conditions (**Figure 3(C)**). Importantly, the studied fungus's fluffy growth was observed under complete dark conditions; however, there was no sign of sporulation under this condition. Besides, the mycelial growth rate was comparatively slower although a significant amount of spore formation was noticed under 12h/12h alternate light and dark conditions. Our results are partially agreed with the earlier works of [18], who reported the best growth of fungi (*Pestalotiopsis fici*, *Pestalotiopsis guepinii* and *Pestalotiopsis palmarum*) under the dark condition; although alternating 12 hours light plus

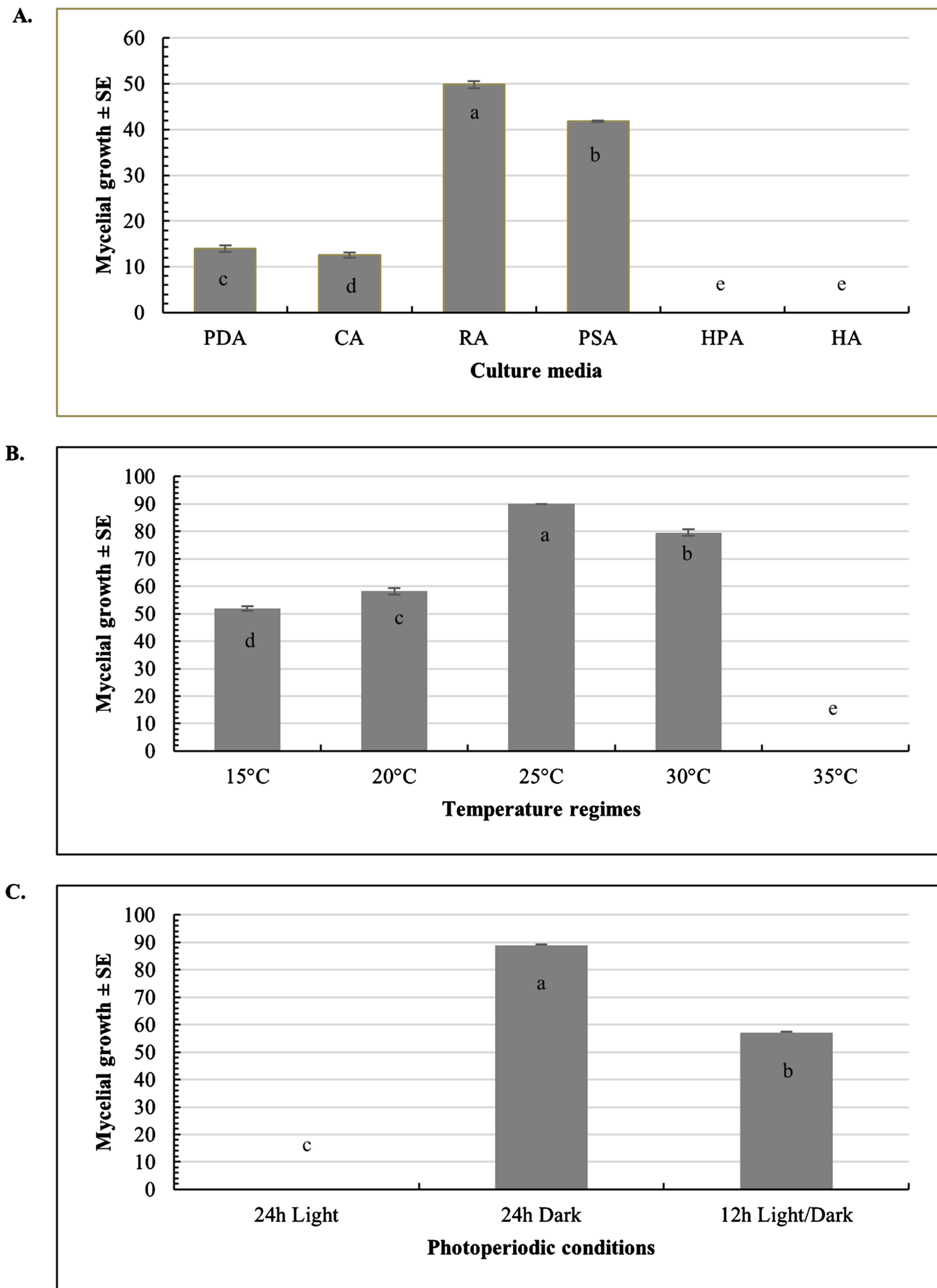
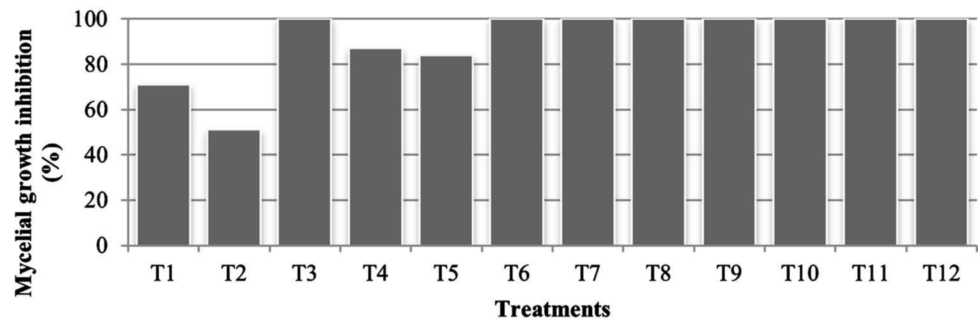


Figure 3. Effect of fungal culture media, temperature and pH on mycelial growth of *P. theae* after 7 dpi. Data represents mean ± standard error of six replicates.

12 hours darkness produced alternative rings between fluffy and abundant in cultures growth. In another lab bioassay, *Pestalotiopsis microspora* was isolated from diseased tissues of oil palm and incubated on PDA at 25˚C under dark conditions [18] [21].



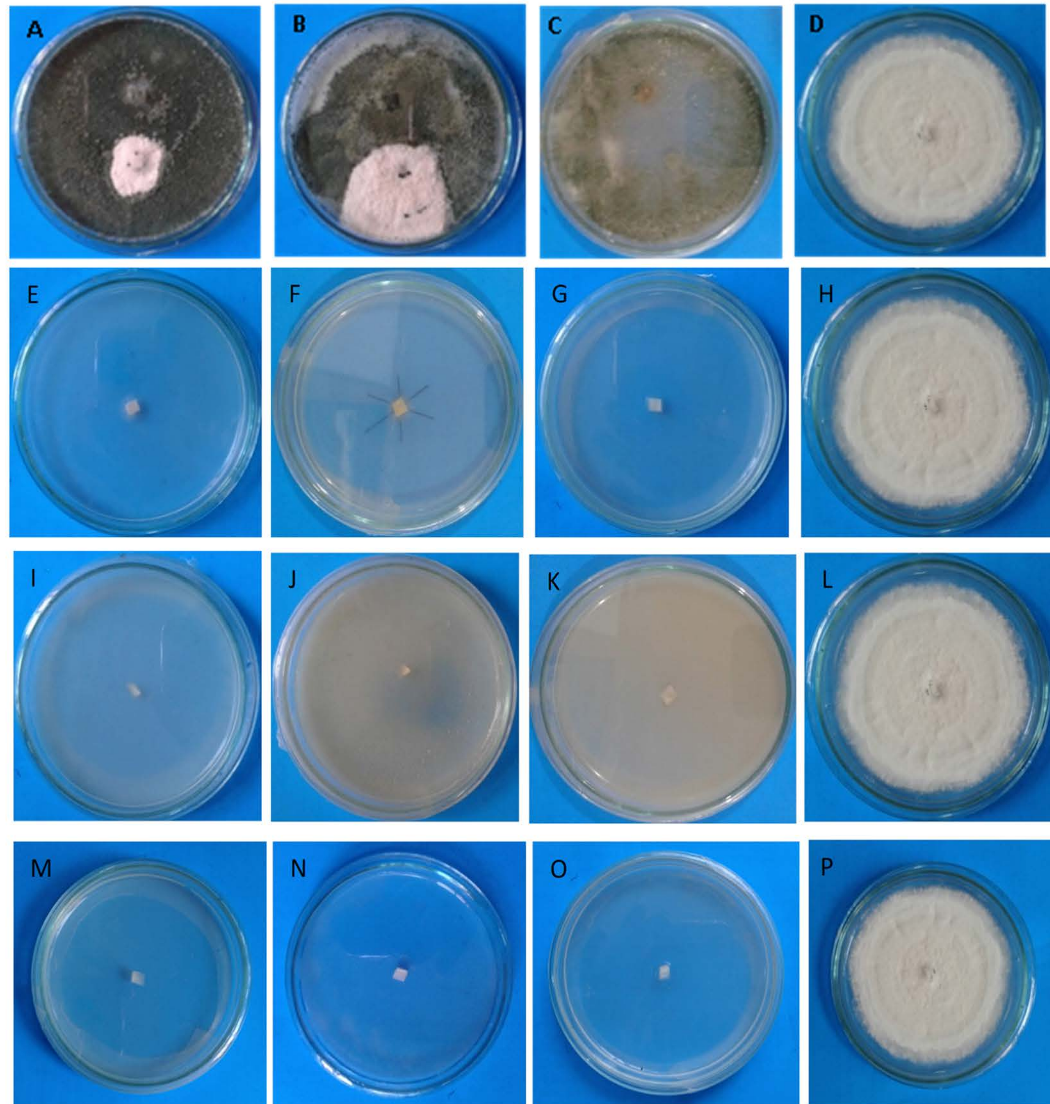


Figure 4. Effect of bio-control agents and fungicides on mycelial growth of *P. theae* at 7 dpi. Here, T1—*T. reesei*; T2—*T. harzianum*; T3—*T. asperellum*; and T4—Amistar Top 250 ppm; T5—Amistar Top 500 ppm; T6—Amistar Top 750 ppm; T7—Ridomyl Gold 250 ppm; T8—Ridomyl Gold 500 ppm; T9—Ridomyl Gold 750 ppm; T10—Tilt 250EC 100 ppm; T11—Tilt 250EC 250 ppm; T12—Tilt 250EC 500 ppm. (A) *T. reesei* vs *P. theae*; (B) *T. harzianum* vs *P. theae*; (C) *T. asperellum* vs *P. theae*; (D) Control (*P. theae*); (E) Amistar Top 250 ppm; (F) Amistar Top 500 ppm; (G) Amistar Top 750 ppm; (H) Control (*P. theae*); (I) Ridomyl Gold 250 ppm; (J) Ridomyl Gold 500 ppm; (K) Ridomyl Gold 750 ppm; (L) Control (*P. theae*); (M) Tilt 250EC 100 ppm; (N) Tilt 250EC 250 ppm; (O) Tilt 250EC 500 ppm; and P: Control (*P. theae*).

3.3. Effect of Bio-Control Agents and Fungicides on the Targeted Fungus

In the current study, very promising results were found concerning the restriction of the vegetative growth of the targeted fungus by bio-control agents (**Figure 4**). Different bio-control agents were showed a varying degree of growth inhibition. Fungal pathogen—*P. theae* was completely inhibited by *T. asperellum*, followed by 70% mycelial inhibition was found due to *T. reesei* and the least 50% inhibition of vegetative growth was observed by *T. harzianum*. In a lab bioassay, it was reported that bio-control agents can produce water-soluble and volatile metabolites and act against soil-borne fungal pathogens [26] [27]. *Trichoderma* spp. was found as an excellent bio-control agent against fungal pathogens [16]. Furthermore, a group of researchers obtained the promising inhibition of mycelial growth of *Fusarium solani* by the bio-control agents *Trichoderma* spp. [12].

In the present investigation, Ridomil gold (Chemical group: 64% Mancozeb 4% Metalaxyl-M) gave an excellent mycelial growth inhibition of the targeted fungus and completely inhibited the mycelial growth of *P. theae* in all concentrations (**Figure 4**). Like Ridomil gold, fungicide-Tilt 250EC (Chemical group: Propiconazole) showed the best inhibition of mycelial growth of the targeted fungus, where *P. theae* was completely inhibited by all of the three concentrations (100 ppm, 250 ppm and 500 ppm). Although the lower concentrations (250 ppm and 500 ppm) of Amistar top (Chemical group: Azoxystrobin + Difenoconazole) inhibited the mycelial growth of the fungus over 80%. Importantly, the higher concentration (750 ppm) of Amistar top could restrict the mycelial growth of *P. theae* completely. Our results conform to the findings of other researchers who reported propiconazole as an effective fungicide against *Pestalotiopsis guepinii* [28] [29]. It has been reported that fungicides Tilt 250EC and Amistar Top 325 SC were effective against the mycelium growth of *Curvularia lunata* [16]. Similarly, scientists have evaluated several concentrations of Ridomyl Gold MZ 68 Wg, Tilt 250 EC and Amistar Top 325 SC fungicides where Tilt 250 EC was effective against the mycelium growth of *Fusarium solani* [12].

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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