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First report of pre-harvest amla fruit rots disease caused by *Pestalotiopsis* sp. in Bangladesh

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Abstract

Fruit rot disease of Amla (*Phyllanthus emblica* L.) reduces the quality of the fruits and causes economic loss. An investigation was conducted to find out pre-harvest fruit rot fungal pathogen associated with Amla fruits. The fungal pathogen was isolated using tissue planting method. Both morphological and molecular characterization confirmed the pathogen identity as *Pestalotiopsis* sp. Result indicated that *Pestalotiopsis* sp. showed the highest radial mycelial growth on potato dextrose agar (PDA) medium. The utmost vegetative growth of the identified fungus was recorded at 25°C on PDA medium, however, a range of temperature may be suitable for the fungal growth. Sodium benzoate and vinegar were tested against the fungus for food preservative. Sodium benzoate at 100mM concentration exhibited efficiency to inhibit the radial growth of the fungus. Chemical food preservatives-sodium benzoate could be used to control the growth of *Pestalotiopsis* sp., associated with Amla fruit rot disease. To the best of our search, quite a few attempts have been taken to investigate the fruit rot disease of Amla. Therefore, fruit rot disease caused by *Pestalotiopsis* sp., is the first record in Bangladesh.

Keywords: Fungal disease, Molecular identification, Fungal biology, Preservatives.

INTRODUCTION

Amla (*Phyllanthus emblica* L.) is a commonly used medicinal plant belonging to the family Euphorbiaceae which fruits contain a higher content of nutritional properties. It is a natural plant used in herbal preparations and almost all parts of the plant including the fruit, seed, leaves, root, bark and flowers are used for medicinal purposes (Gantait *et al*., 2021; Lim, 2012). Amla has been originated in India and intensively cultivated in different tropical and sub-tropical countries in the world (Thilaga *et al*., 2013). In Bangladesh, it is cultivated in evergreen, semi-evergreen, sal and homestead forest. Amla has a significant role in Ayurveda and it is also used as a prospective food additive or in the nutraceutical and pharmaceutical industries (Dasaroju and Gottumukkala, 2014). This plant is used as anti-aging, anti-diabetic, anti-oxidant, anti-ulcerous, cardio-protective, hepato-protective, memory enhancing and immuno-modulatory (Gantait *et al*., 2021). Although having anti-microbial functions, high quantities of sugar content with other nutrient and low pH level make the fruit susceptible to fungal infection (Singh & Sharma, 2007). A number of pre-harvest and post-harvest diseases are known to cause significant economic losses. Pre-harvest fruit diseases occur in the month of January and post-harvest fruit diseases occur due to not properly harvesting and handling. Fungi not only

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decay, deteriorate or reduce economic loss but also produce mycotoxins, which has been a serious concern for human health (Moss, 2002). Recently, Sikder *et al*. (2020) reported *Thielaviopsis paradoxa* as a pre-harvest fungal pathogen causing black spot disease of Amla fruit in Bangladesh. Sengupta *et al*. (2020) reviewed post-harvest diseases of Amla such as fruit rot, anthracnose and blue mold along with its management strategies in India. The diversity of fungal diseases of Amla fruits have been investigated and recorded a number of fungi associated with the fruit disease such as rot, blue mold and green mold. *Pestalotiopsis* sp. was recorded as an important pre-harvest pathogen, which is responsible for Amla fruit rot disease (Sharma & Sharma, 2018; Verma & Verma, 2015). The present investigations were undertaken to isolate and identify the fungus associated with pre-harvest fruit rot disease of Amla using morphological and molecular characterization; to evaluate the growth characters on different culture media and temperature conditions of the isolated fungus; and to assess the fungitoxicity of chemical food preservatives against the radial growth of the fungus.

MATERIALS AND METHODS

Isolation and Identification of the fungus: Diseased amla fruits were collected from the Wildlife Rescue Centre (23°52'11"N; 90°16'04"E) at Jahangirnagar University, Bangladesh. Collected samples were separately transferred in a sterile zipper bag. Tissue planting method was followed to isolate fungal pathogen from the infected amla fruits (Mallik *et al*., 2021).

The fungal pathogen was identified based on colony morphology, mycelial and conidial characteristics as well as growth behaviors. Conidia were observed in 10-days old culture and described based on the color of conidial masses, shape, septation and basal and apical cell of conidia, conidiophores in the mycelium and presence or absence of chlamydospores (Lazarotto *et al.,* 2014).

Molecular identification was carried out by using the ITS sequence of the fungal genome (Sikder *et al*., 2020). The fungal genomic DNA was extracted and quantified (NanoDrop Spectrophotometer, ND2000, Thermo Scientific, USA). According to White *et al.* (1990) the ITS region of the selected fungus was amplified using universal primers ITS4 and ITS5. The PCR was performed in a total volume of 25µl reaction mixture by using 5µl DNA template (20ng/µl) and GoTaq® G2 Hot Start Green Master Mix (Promega, USA) as pre-heat at 94℃ for 5 min, 35X (94℃ for 30 s, 57℃ for 30 s, 72℃ for 5 min), and 72℃ for 10 mins (Sultana *et al*., 2020). The purified amplicons of approximately 650 bp was sequenced under Sanger sequencing platform. Sequencing data were assembled by GeneDoc and sequence alignment was checked with Chromas. After necessary trimming from both ends of the DNA sequence, data were sent to NCBI and received accession number. A BLAST search with the ITS sequences was used to reveal the closest matching taxa in Pestalotiopsidaceae (Senanayake *et al*., 2015). Multiple sequence alignments were performed using MEGA6 software (Tamura *et al*., 2013). Maximum likelihood, Maximum parsimony, and Neighbor-joining trees were generated (Tamura *et al*., 2013).

Pathogenicity test: For *in vitro* pathogenicity test, Amla fruits were washed with tap water, rinsed with distilled water and surface-sterilized with 70% ethanol. Four to five fruits were wounded with sterilized needle and for control wounded Amla was placed without any inoculation of the fungi. The inoculated Amla fruits with isolated fungus and the controls were kept in a desiccator in laboratory conditions. Each of the fruits was moistened with wet tissue to create a humid condition and kept for further development of inoculated fungi. After the development of symptoms with fungal mycelium, these were transferred into PDA medium and observed until the growth of mycelium and formation of spore of the fungal pathogen. Morphological examination of the re-isolated fungus showed the same characters as primarily isolated fungus which proved that the pathogenicity test was successful.

Effect of culture media, temperature and food preservatives: Six different culture media such as potato dextrose agar (PDA), carrot agar (CA), honey peptone agar (HPA), Richard agar (RA), potato sucrose agar (PSA) and Hansen's agar (HA) media were selected to investigate the growth characteristics of the isolated fungus (Sikder *et al*., 2019). To find out the effect of temperature on fungal mycelial growth, the tested fungus was inoculated on PDA medium and incubated at 15^0C, 20^0C, 25^0C, 30^0C and 35^0C for 7 days (Ahmmed *et al.,* 2020; Shamoli *et al.*, 2013). According to Rahman *et al*. (2015) two food preservatives *viz.*, sodium bicarbonate and acetic acid (vinegar) at three different concentrations *viz.*, 50mM, 75mM and 100mM were evaluated against the fungus.

Data analysis: Normality and homogeneity of variance were checked for the data generated during the experiment. Data on the effects of fungal culture media, temperature, food preservatives on the vegetative growth of fungus was found to be normal and statistical analysis were performed with Duncan's Post-Hoc test (SPSS-16).

RESULTS AND DISCUSSION

Identification of the pathogen and test of pathogenicity: *Pestalotiopsis* sp. grew on Amla fruits as a pre-harvest fruit rot pathogen which produced a black color spot with a brown margin. A white-colored fungal colony was observed on PDA medium and sclerotium formed with aged. Acervuli scattered, globose to lenticular, raised. Three median cells with pigmentation in which two apical cells are light brown in color and the middle one was dark brown in color (Fig. 1). Flagella were present on both ends of the conidium. Above mentioned characters suggest that the studied fungus was *Pestalotiopsis* sp. Maharachchikumbura *et al.* (2011) and Barbu *et al.* (2018) identified *Pestalotiopsis* sp. based on morphological characteristics. Besides Bhuiyan *et al*. (2021) studied and identified *Pestalotiopsis* sp. causing leaf spot disease of coconut through morphological characterization.

A BLAST search with ITS sequence of the fungal pathogen showed 98 to 99% identity with *Pestalotiopsis* sp. which were previously deposited in the NCBI database. Maximum likelihood (ML), Neighbor-joining (NJ), Maximum parsimony (MP) trees were generated and all of the trees showed similar topology. In this article, only ML tree have been

presented. There were three major clades; clade-I consists of 12 taxa with different species under the genus-*Pestalotiopsis* and *Neopestalotiopsis* with 100% bootstrap value in which our studied organism occupied **(**Fig. 2**)**. Clade-II consists of 6 taxa from genus-*Pseudopestalotiopsis* with 75% bootstrap value. Clade-III has 17 different species of *Pestalotiopsis* with 99% bootstrap value. Based on the phylogenetic tree, our studied organism showed closeness and form a cluster with the species of Clade-I which indicates that it belongs to *Pestalotiopsis* sp. Sikder *et al.* (2020) identified *Thielaviopsis paradoxa* causing black spot disease of Amla fruits by molecular technique. Bhuiyan *et al*. (2021) successfully conducted an experiment to identify *Pestalotiopsis* sp. through molecular technique.

In our experiment, *in vitro* pathogenicity test was conducted using the detached fruit inoculation technique. Characteristic symptom was reproduced and the same pathogen was re-isolated from the artificially inoculated fruit to confirm Koch's Postulates and the further investigation ensured the pathogenic identity of the fungus.

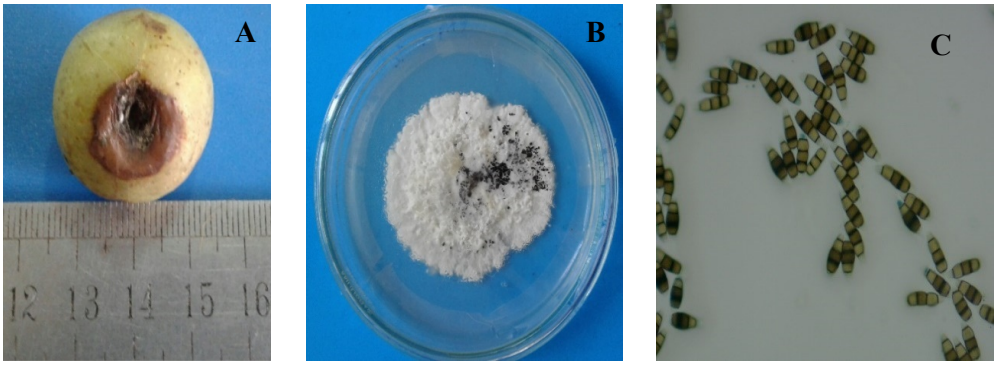

Fig. 1. Morphology of *Pestalotiopsis* sp. causing pre-harvest fruit rot disease on Amla fruits. A: Symptoms of fruit rot disease; B: Vegetative growth of *Pestalotiopsis* sp., C: Conidia of *Pestalotiopsis* sp. (10 X 10x)

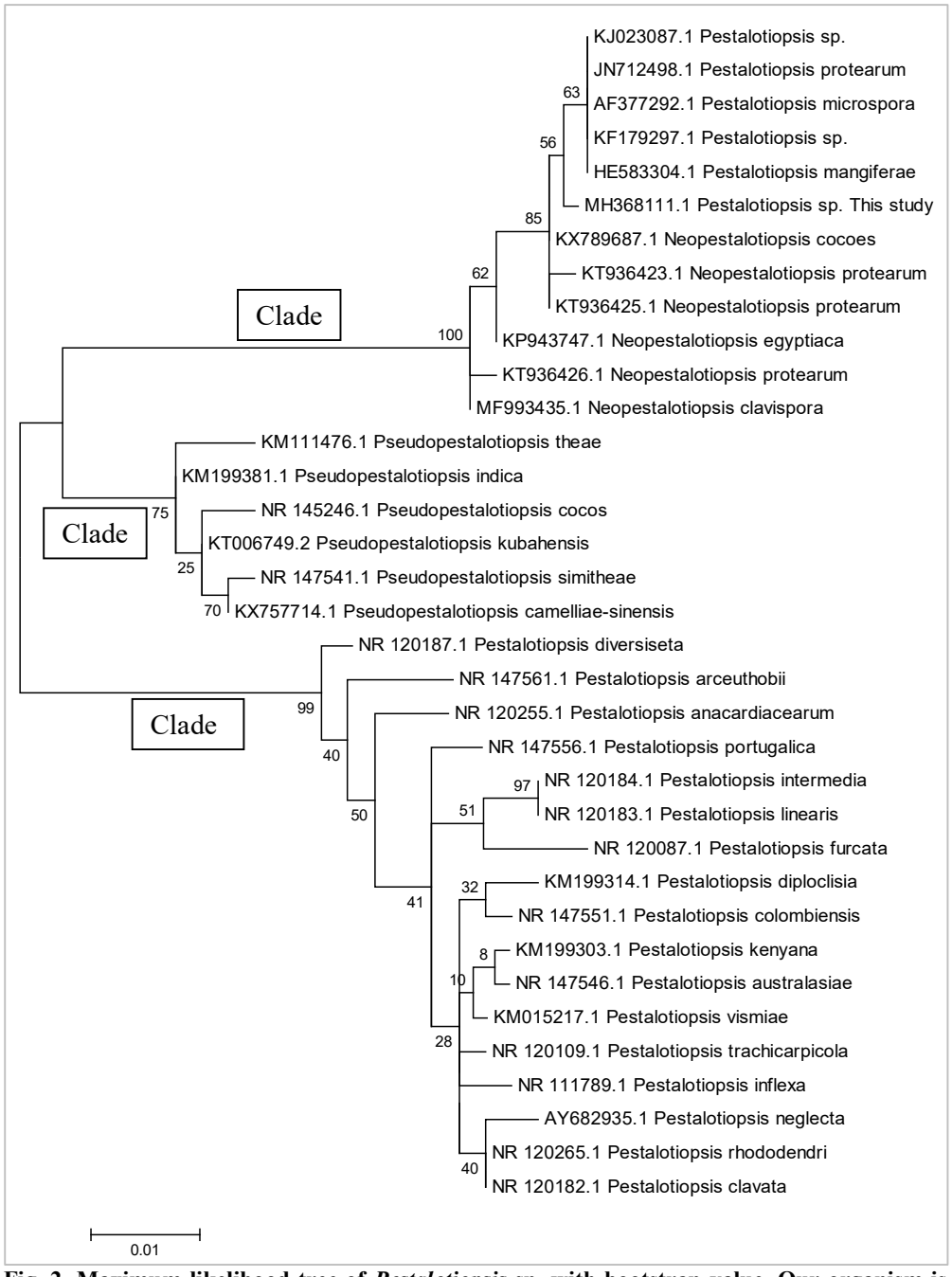

Fig. 2. Maximum likelihood tree of *Pestalotiopsis* sp. with bootstrap value. Our organism is marked as "This study" (Accession no. MH368111.1)

Growth characteristics of the fungus: Growth media along with other physiological parameters are an important factor to study on fungal biology which manipulates the morphological and reproductive growth and development of the fungi (Kim *et al*. 2005). PDA, PSA, CA, RA, HPA and HA were used as culture media for the vegetative growth of *Pestalotiopsis* sp. The statistical differences among the fungal culture media to support the growth of tested fungus were found (Fig. 3). *Pestalotiopsis* sp. showed their highest mycelial growth on PDA medium, followed by RA while the growth observed lowest on both HPA and HA medium. In our experiment, *Pestalotiopsis* sp. was able to grow on most of the culture media tested. EL-Gali (2017) found that *Pestalotiopsis fici* showed the maximum mycelial growth on PSA medium. Fovo *et al*. (2017) experimented on the effect of three culture media *viz.*, PDA, malt extract agar and V8 juice agar on the growth of *Pestalotiopsis microspora* and the fastest mycelial growth were found on V8 juice agar medium. Majumdar *et al*. (2019) intensively investigated the growth and sporulation of *Pestalotiopsis mangiferae* on different fungal culture media and observed the highest mycelial growth on malt extract agar medium and sporulation on PDA medium. Bajo *et al*. (2008) reported the optimum growth rate of *Pestalotiopsis funerea* on TAKAY medium compared to other four different media due to the fact that TAKAY medium consists of many compounds and nutrients.

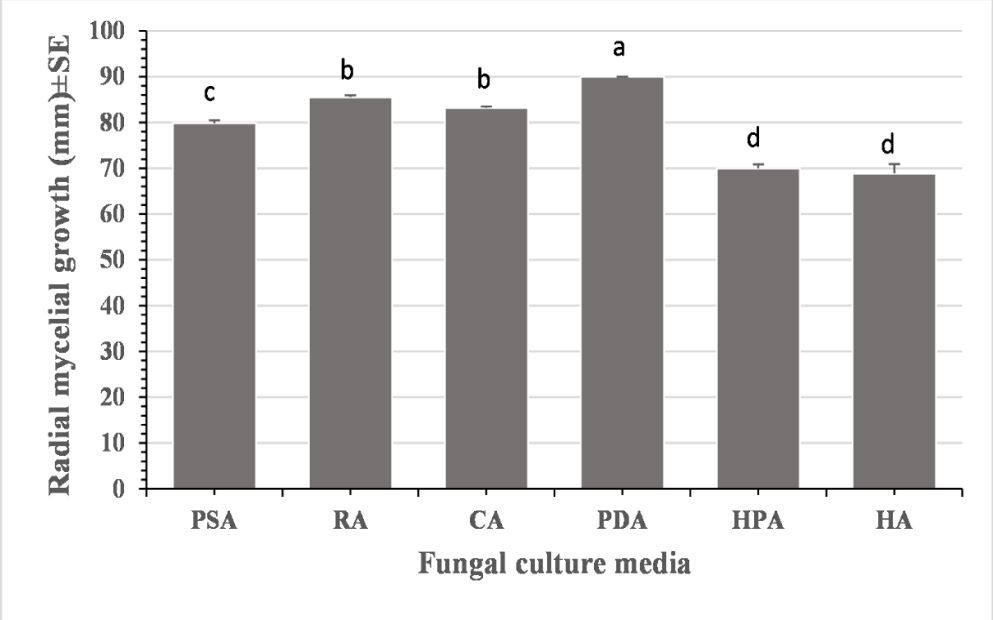

Fig. 3. Effect of culture media on vegetative growth of *Pestalotiopsis* sp. at 7 dpi. Data represent mean ± standard error (SE) of nine replications; PSA, potato sucrose agar; RA, Richard agar; CA, carrot agar; PDA, potato dextrose agar; HPA, honey peptone agar; HA, Hansen's agar

Pestalotiopsis sp. grew well at all the temperatures tested (**Fig. 4**) but a range of temperature 20º C to 30ºC may be preferred by the pathogen. The maximum vegetative growth was recorded at 25ºC. These results are in conformity with the findings of Bhuiyan *et al*. (2021) who found that *Pestalotiopsis* sp. grew very well at 25ºC and the growth almost retard at 15ºC and 35ºC. Likewise *Pestalotiopsis* spp. (*Pestalotiopsis fici, P. guepinii* and *P. palmarum*) had the utmost growth at 25ºC temperature (El-Gali, 2017). Similarly, Fovo *et al*. (2017) investigated the effect of temperature on the vegetative growth of *Pestalotiopsis microspora* which showed 23ºC as an optimum temperature and at 33ºC, the growth was almost ceased.

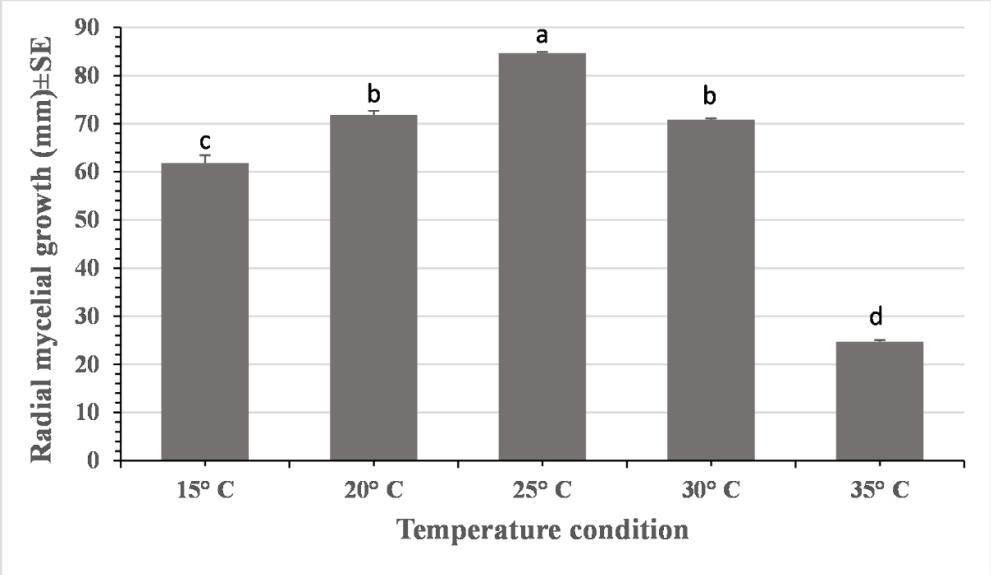

Fig. 4. Effect of temperature on vegetative growth of *Pestalotiopsis* sp. at 7 dpi.

Two commonly used food preservatives were implemented and found that there were trends of vegetative growth inhibition (%) of the isolated fungus with increasing concentrations of sodium benzoate and acetic acid (Fig. 5-6**)**. The utmost mycelial inhibition (%) of *Pestalotiopsis* sp. was recorded due to the higher dose of the two chemical preservatives. Although, the lower dose of sodium benzoate was enough to stop the vegetative growth of the isolated fungus to a certain extent. However, the higher dose of sodium benzoate was necessary for complete mycelial growth inhibition of *Pestalotiopsis* sp. (Fig. 5-6).

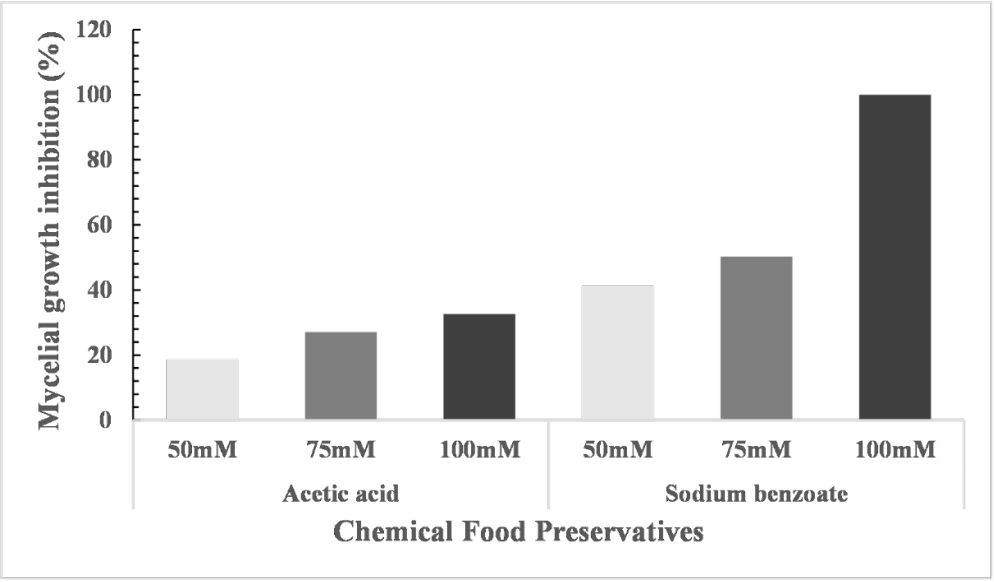

Fig. 5. Effect of chemical food preservatives on vegetative growth of *Pestalotiopsis* sp. at 7dpi.

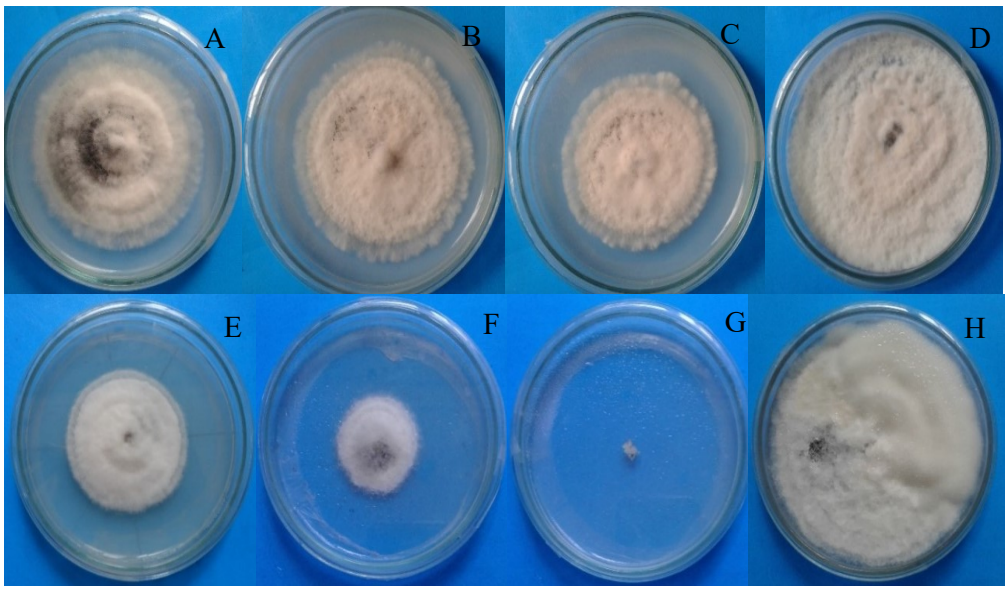

Fig. 6. Effect of chemical food preservatives on vegetative growth of *Pestalotiopsis* sp. at 7 dpi. A, 50mM vinegar; B, 75mM vinegar; C, 100mM vinegar; D, Control; E, 50mM sodium benzoate; F, 75mM sodium benzoate; G, 100mM sodium benzoate; H, Control.

Matny and Al-Rawi (2012) reported vinegar (acetic acid) as the most effective to inhibit fungi and 2% of acetic acid inhibited 62% of *Penicillium digitatum*. Likewise, Lind *et al*. (2005) evaluated the antifungal potentiality of vinegar against several saprophytic and pathogenic fungi in which the minimal inhibitory concentration values of vinegar were established for all fungi and suggested that acetic acid was a potent antifungal organic acid (Rogawansamy *et al.*, 2015; Peláez *et al*., 2012; Shi *et al.,* 2016). Concentration of 0.05% (w/v) of sodium benzoate showed the complete inhibition of *Aspergillus niger* and *Penicillium citrinum* (Nwafor & Ikenebomeh, 2009). Similarly, Ogiehor and Ikenebomeh (2004) reported the efficacy of sodium benzoate against the several species of *Aspergillius* sp. Moreover, Alsudani (2017) showed that the sodium benzoate had very high inhibitory activity against the mycelial growth of several food spoilage fungi.

Conclusion: Amla fruit is an important source of vitamin C to fulfill our daily requirements. But the fruit infected with fungal pathogens may cause mycosis. So, it's a pressing need to introduce effective strategies to control the fungal disease of the fruit. Taking human health hazards into consideration, the findings of the present experiment will facilitate the way of study on the pre-harvest fruit rot disease of *Phyllanthus emblica* L. Data on the effect of culture media, temperature and food preservatives will help future researchers to innovate novel strategies to combat against the pre-harvest disease of amla fruit.

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