J. Bio-Sci. 32(1): 01-12, 2024 **ISSN 1023-8654**
http://www.banglajol.info/index.php/JBS/index
DOI: doi.org/10.3329/jbs.v32i1.74983

FIRST REPORT OF FUSARIUM FRUIT ROT CAUSING *FUSARIUM INCARNATUM-EQUISETI* ON AMLA IN BANGLADESH

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Abstract

Amla (*Phyllanthus emblica* L.) fruit is prone to rot, which is responsible for the qualitative losses of the fruits both during the pre- and post-harvest period. A fungal pathogen was isolated using tissue planting methods. The genomic DNA of the isolated fungus was amplified and sequenced under the Sanger Sequencing platform. Blast search showed 99% sequence similarity with *Fusarium incarnatum-equiseti* species complex. The vegetative growth of the targeted fungus was assessed on several fungal culture media, in which Richard Agar medium exhibited the maximum mycelial growth at 25ºC temperatures. Neutral to slightly alkaline condition was mostly preferred by this fungus. Among three light regimes, the complete light condition was ideal for the mycelial growth of the fungus. Three aqueous plant extracts were evaluated to check *in vitro* vegetative growth, in which garlic (20%) was quite enough to restrict complete mycelial growth of it. Two food preservatives were also assessed, where sodium benzoate (50 mM) was most effective for the restriction of the targeted fungus compared to vinegar. Therefore, garlic and sodium benzoate could be used to manage the fungal growth of *F. incarnatum-equiseti* associated with Amla fruits. To the best of our knowledge, Amla fruit rot caused by *F. incarnatum-equiseti* is the first record in Bangladesh.

Key words: Garlic, light, molecular analysis, pH, sodium benzoate, turmeric, vinegar.

Introduction

Amla (*Phyllanthus emblica* L.) is one of the common fruits with its greater content of vitamin C, flavonoids, and other phytochemicals. It is used in Ayurvedic medicine, including fruit, seed, leaves, root, bark, and flowers (Lim 2012). Although Amla has been cultivated in arid and semiarid regions, it is abundant all over Bangladesh. The fruit contains antioxidant properties and is being used to treat many human diseases (Mishra and Sharma 2017). However, fruits are known to attack by fungal pathogens due to low pH and higher sugar contents (Singh and Sharma 2007). Both parasitic and saprophytic fungi are known to cause fruit spots and rotting. For instance, fruit rot, anthracnose, blue-, green- and yellow mold diseases have been reported in India (Verma and Verma 2015). Akhund et al. (2010) found 43 fungal species in India, which cause surface infection on amla fruits. In Bangladesh, several fungal pathogens have been reported to cause disease and rot of Amla fruits, such as *Thielaviopsis paradoxa* (Sikder et al. 2020); *Pestalotiopsis* sp. (Sultana et al. 2022), *Aspergillus terreus*, *Aspergillus aculeatus*, *Penicillium rolfsii*, *Penicillium herquei*, and *Mucor* sp. (Sultana et al. 2023). These fungi are responsible for qualitative, quantitative, and economic losses. Fungi also generate mycotoxins, which is a serious health concern for both wild animals and human beings (Moss 2002). For instance, *Fusarium equiseti* is known as a major source of mycotoxins such as trichothecenes (diacetoxyscirpenol, nivalenol) and zearalenone (Barros et al. 2012). The present

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investigation was conducted to identify the fungus pathogen associated with pre-harvest amla fruit rot; to investigate the effect of pH, light, and culture media on the mycelial growth; and to assess the fungitoxicity of selected phytoextracts and food preservatives against isolated fungus.

Materials and Methods

Sample collection, isolation, and identification of the fungus

Diseased samples collected from trees located in the studied area Wildlife Rescue Centre of Jahangirnagar University (N: 23°52'19"; E: 90°16'28"). Those disease samples were brought to the Laboratory of Mycology and Plant Pathology, Jahangirnagar University, Savar, Dhaka-1342 for further investigations. The tissue planting method was performed for the isolation of the fungal pathogen from Amla fruits.

The fungal pathogen was identified based on colony morphology, morphological characteristics of conidia such as shape, number of septa and apical and basal cells of macroconidia, the appearance of conidiophores in the aerial mycelium, and presence or absence of chlamydospores (Leslie and Summerell 2006).

The fungal genomic DNA was isolated using a commercial kit (Promega, USA) and DNA concentration was quantified using NanoDrop Spectrophotometer (Thermo Scientific, USA). The ITS region of the rDNA of the isolated fungus was amplified using universal primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAG G-3'). The polymerase chain reaction (PCR) was done in a 25µl reaction mixture, which contains a DNA template (20ng), primer sets, GoTaq® G2 Hot Start Green Master Mix (Promega, USA), and sterile water. The PCR cycles were conducted at 94ºC for 5 minutes, 35 cycles of denaturation at 94ºC for 45 seconds, annealing at 52ºC for 30 seconds, extension at 72ºC for 5 minutes, and final extension at 72ºC for 10 minutes (Sikder et al. 2019). The gel run of the PCR product was carried out (CBS Scientific, USA), and photographs were taken. The amplicons were purified (Maxwell® 16 DNA Purification Kit, Promega, USA) and the purified PCR product was sequenced in the Sanger sequencing platform (*First BASE* Laboratories Sdn Bhd, Malaysia). Sequencing data were assembled, checked for artifacts, and compared in NCBI Genbank. The phylogenetic analysis was conducted using the multiple sequence alignment tools via MEGA 6 software.

Pathogenicity

For pathogenicity, Amla fruits were washed properly with running tap water, followed by washing with distilled water and 70% ethanol (Sultana et al. 2022a). Using a sterile needle, Amla fruits were injured and isolated fungus was inoculated into the fruits, whereas inoculated with sterile water served as control. Fruits were moistened with wet tissue paper to create humid conditions. Then, those Amla fruits were kept in a sterile desiccator. After a week, characteristic symptoms developed, and the infected portions were transferred to the PDA medium. After the incubation period, fungal spores were examined under the microscope.

Effect of culture media, light, and pH on the vegetative growth of the fungus

Six different culture media i.e. Potato dextrose agar (PDA), Carrot agar (CA), Richard agar (RA), Honey peptone agar (HPA), Potato sucrose agar (PSA) and Hansen's agar (HA) media were prepared to assess the mycelial growth of isolated pathogen (Ahmmed et al. 2021). The effect of light conditions on the mycelial growth of the isolated fungus was conducted in which white tube light was used for light treatment in the

growth chamber and brown paper was used to wrap the petri plate to create darkness (Ahmmed et al. 2022). The inoculated plates were incubated under the tube lamp (at two-foot height) at a temperature of 25±2ºC. The inoculated plates were subjected to light exposure as follows: (a) continuous light i.e., 24 hours light condition, (b) continuous dark i.e., 24 hours dark condition, and (c) alternating 12 hours light and dark condition. To know the effect of hydrogen ion concentration (pH) on the growth and development of fungus, the PDA medium was adjusted to pH 5, 6, 7, 8, and 9 with the addition of 1N NaOH or lactic acid before autoclave (Sultana et al. 2022). After inoculation of isolated fungus on PDA each petri-plate and this petri dish were incubated at 25ºC for 7 days. The measurement of mycelial development under different pH conditions was done using an mm scale.

Effect of phyto-extracts and food preservatives on the mycelial growth of the fungus

Black cumin (*Nigella sativa* L.), turmeric (*Curcuma longa* L.), and garlic (*Allium sativum* L.) were used as plant extracts for inhibition of the isolated fungus. The 10%, 20%, and 30% of plant extracts were used for an assessment of efficacy on the vegetative inhibition of the isolated fungal pathogen using the food poison technique (Ahmmed et al. 2020). Besides, vinegar (acetic acid) and sodium benzoate were also used as chemical preservatives against tested fungus. These preservatives were diluted with distilled water and measured as mM (millimolar). The different concentrations of vinegar and sodium benzoate *viz*. 50 mM, 75 mM, and 100 mM were used against the targeted fungus. These chemical preservatives were amended with the PDA medium, the isolated fungal pathogen was inoculated on PDA media both containing chemical preservatives or water only (served as a control) and the mycelial growth inhibition percentage was measured after 7 days of inoculation (Akter et al. 2022).

Data analysis: Data were analyzed using one-way ANOVA, followed by Duncan's post-Hoc test in SPSS-16.

Results and Discussion

Identification of the fungal pathogen

Dark brown to black spots appeared on Amla fruits, and later started rotting (Fig. 1A). These spots further showed pinkish in color. The fungal colony was brown on PDA media (Fig. 1B). The mycelium was hyaline and branched. The conidia were hyaline, septate, and usually falcate in shape (Fig. 1C). The macroconidia were long, fusoid, slender, multi-septate, and slightly curved, or bent at the point of end. Some conidia are intermediate, two or three-celled, oblong, or slightly curved. The microconidia were not observed. These characteristics suggested the fungus was *Fusarium* sp. To confirm the fungal identity at species rank, molecular characterization was performed. Our fungal organism (MH368100.1) showed 99 to 100% sequence similarity with the previously deposited fungal pathogen *Fusarium incarnatum-equiseti* species complex (FIESC) (KY031974.1, HM008677.1, MH290471.1, MH290470.1, KX576658.1). Besides, the currently studied fungus clustered with FIESC with a 97% bootstrap value (Fig. 2). The artificial inoculation of the fungus showed similar symptoms, further confirming the pathogenic nature of the fungus.

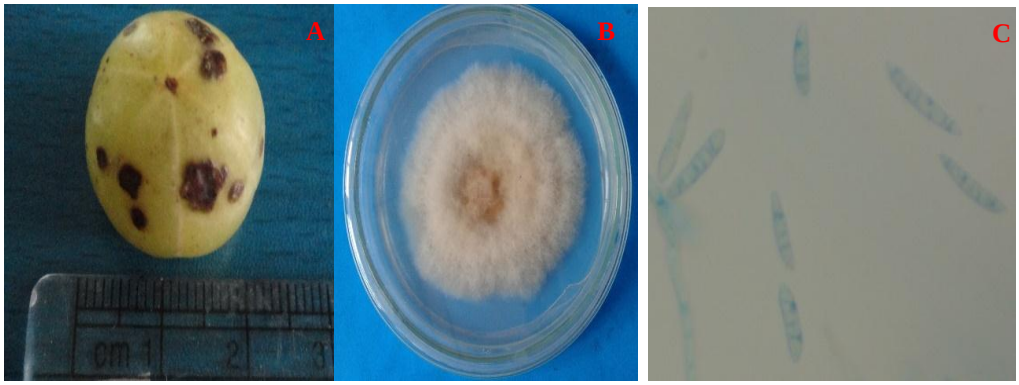

Fig. 1(A-C): Morphological characteristics of FIESC causing fruit rot disease on Amla fruits. A: Symptoms of FIESC, B: Vegetative growth of the fungus on PDA medium, C: Microscopic view of macroconidia and mycelium (10 × 10X).

Growth characteristics of the isolated fungus

The media components are important criteria for fungal culture and study, along with important physiological parameters that lead to maximum sporulation in fungus. Osono and Takeda (1999) stated that fungal culture media having lower glucose content suppresses the overgrowth of fast-growing species and induces sporulation; hence, this medium was useful for fungal identification. Fungal systematics is still based mainly on morphological criteria as observable characteristics. Hence, fungi are recognized and identified basically by their phenotypes (Zain et al. 2009).

The effect of six different solid media on the mycelial growth of FIESC was assessed to know their growth pattern. The results showed that FIESC attained its highest mycelial growth (82.17 mm) on the RA medium, followed by PDA and PSA media, and the lowest mycelial growth (59.83 mm) on the HPA medium (Fig. 3A-B). The fungal colony was whitish on PSA, greyish with a brownish center on the RA, greyish on CA and PDA media; pinkish on HPA and HA media (Fig. 3A). The present results are supported by the previous findings of Mallik et al. (2021) who noted the 78.6 mm, 75.8 mm, and 74.0 mm mycelial growth of *Fusarium solani* on RA, PSA, and CA media, respectively. Besides, Ahmeed et al. (2021) reported that RA and CA media were suitable for the radial growth of *Fusarium oxysporum.* Chittem and Kulkarni (2008) also found the significant mycelial growth of *F. oxysporum* f. sp. *gerberae* on RA and PDA media.

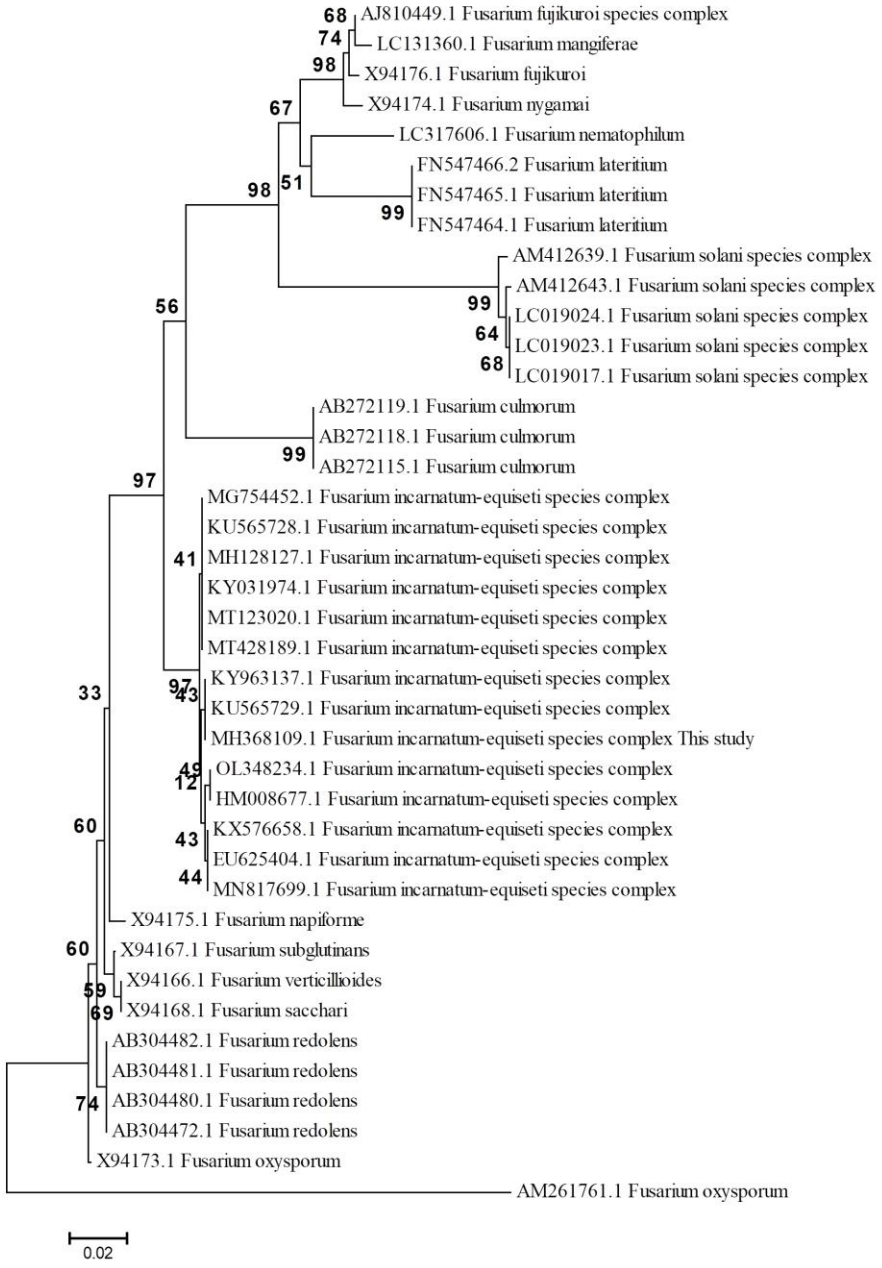

Fig. 2: The dendrogram was derived from the analysis of the ITS sequence dataset of the studied organisms with bootstrap value (Bootstrap replication = 1000). NCBI accession numbers are mentioned before the genus names. Our organism (MH368109.1) is marked with this study.

The variation in pH contents of PDA media correlates with the mycelial growth of almost all the fungi. The suitable pH of media for the best growth and development of test fungus was determined through this part of the experiment. The experimental petriplates were incubated at five different pH conditions *viz*., 5, 6, 7, 8, and 9. The results showed the maximum mycelial growth of FIESC at neutral to slightly saline conditions (Fig. 3C). The growth rate was very slow in acidic conditions (pH 5.0 to pH 6.0). The present findings are supported by previous reports, in which *Fusarium oxysporum* f. sp. *ciceri* (Farooq et al. 2005), *Fusarium oxysporum* f. sp. *ciceri* (Khan et al. 2011) showed the maximum mycelial growth at pH 7.0, *F. oxysporum, F. solani, F. proliferatum, F. sacchari and F. globosum* at pH 7.5; and *Fusarium oxysporum* f.sp. *cumini* at pH 8.0 (Rathore et al. 2015). However, Siddique et al. (2012) reported that *Fusarium oxysporum* f. sp. *phaseoli* exhibited the highest mycelial growth at pH 6.0 to pH 6.5. Likewise, the most suitable growth of different isolates of *Fusarium oxysporum* f.sp. *cubense* was found at a pH level of 5-7 (Somu and Thammaiah 2015). Reports have shown that the growth of fungi could be affected by 'Hydrogen ion concentration' (pH) in a medium in which it grows, either directly by its action on the cell surfaces or indirectly by its effect on the availability of nutrients (Pardo et al. 2006).

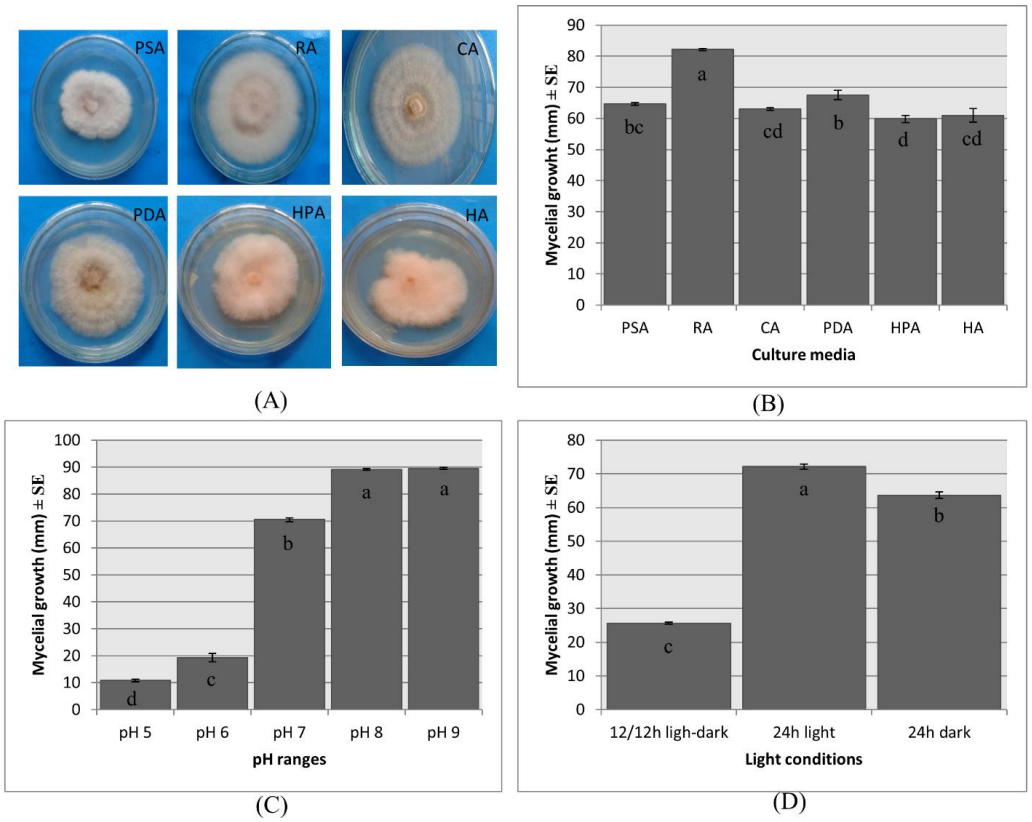

Fig. 3(A-D): Effect of culture media, light, and pH on the vegetative growth (mm) of FIESC. The value represents as mean ± standard error of six replications.

Light is a very important parameter for every living cell and hence, the effect of light on the mycelial growth of the isolated fungus was evaluated (Fig. 3D). The results showed that FIESC had the highest (72.16 mm) vegetative growth under continuous light conditions, followed by complete dark conditions, and lowest under alternative light-dark conditions. Both complete light and dark were suitable for the mycelial growth of FIESC. The radial mycelial growth of all studied fungi (*Fusarium solani, F. oxysporum, F. sacchari, F. proliferatum,* and *F. globosum*) was optimum under complete dark conditions. However, the continuous darkness is not recommended for the mycelial growth or sporulation of the *Fusarium chylamydosporum.* Somu and Thammaih (2015) reported the highest mycelial growth of *Fusarium oxysporum* f. sp. *cubense* (77.40 mm) under alternate light-dark conditions, followed by 70.80 mm in continuous light while the lowest growth of the fungus (65.20 mm) was observed under continuous dark conditions.

Effect of plant extract against targeted fungus

In the present study, increasing trends of mycelial growth inhibition were noticed with increased doses of phytoextract (Fig. 4-5). Although lower concentrations of black cumin and turmeric (10% and 20%) did not show any significant result against tested fungus. Importantly, the higher dose (30%) of black cumin and turmeric extracts showed somewhat positive results against FIESC. It showed that the highest inhibition (51.48%) was recorded by 30% turmeric extract. Aqueous extract of garlic was very promising, in which above 60% mycelial inhibition was found for lower concentrations of garlic extract (10%) and 100% inhibition of the fungus was recorded by both 20% and 30% of garlic extract (Fig. 4-5). Likewise, the positive trends of vegetative growth inhibition were observed with higher doses of black cumin extract. These results indicate that mycelial inhibition levels might increase if higher doses are used in further investigation. Shokri et al. (2012) reported several antifungal compounds such as p-cymene, thymoquinone, trans-anethole, 2-methyl-5(1-methyl ethyl)-Bicyclo[3.1.0]hex-2-en and γ-terpinene. Helen et al. (2012) reported that turmeric had significant antifungal activity against *Aspergillus flavus, Fusarium oxysporum* and *Penicillium chrysogenum*. Similarly, Jayaprakasha et al. (2001) also concluded that turmeric oil had antifungal activity against *Aspergillus flavus, Penicillium digitalum* and *Fusarium moniliforme.* Geraldin (2016) reported that turmeric and garlic extracts showed significant activity against *Alternaria solani*, *Fusarium oxysporum* f. sp. *lycopersici*, *Pythium ultimum* and *Rhizoctona solani*. Furthermore, Wongkaew and Sinsiri (2014) revealed that ethanolic extracts of turmeric were effective against *Alternaria alternata*, *Pythium* sp and *Fusarium oxysporum* f.sp. *lycopersici.* Biochemical analysis showed that the rhizome of turmeric contains aromatic oil turmerones and cucurminoids, which possess an active antimicrobial feature. The lipophilic properties of essential oils allow them to penetrate the plasma membrane, causing polysaccharide accumulation under drought stress conditions and leading to plasmalemma breakage in fungal cells.

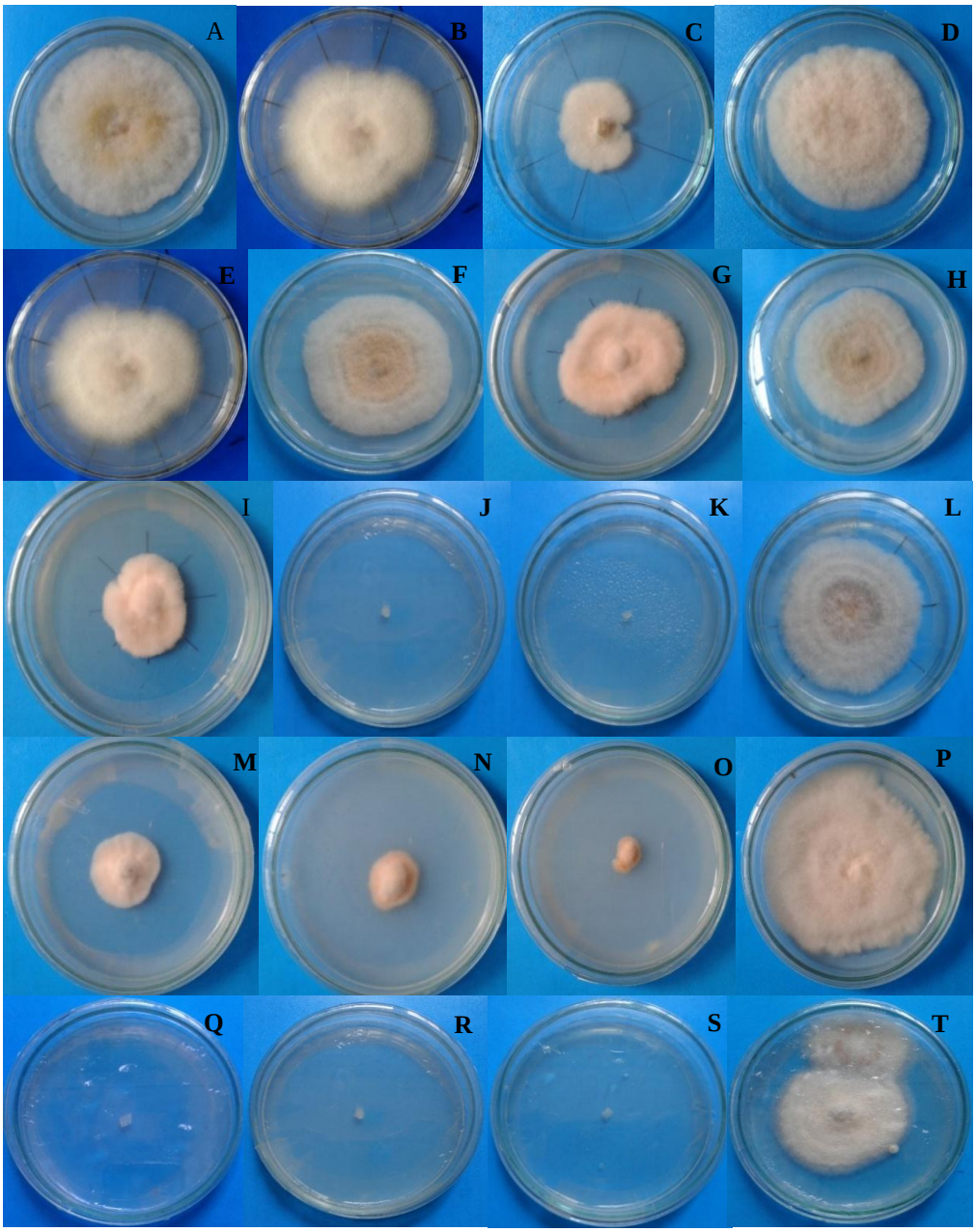

Fig. 4 (A-T): Effect of phytoextracts and food preservatives on mycelial growth of *FIESC* at 7 dpi. A: black cumin (10%); B: black cumin (20%); C: black cumin (30%); D: control; E: turmeric (10%); F: turmeric (20%); G: turmeric (30%); H: Control; I: garlic (10%); J: garlic (20%); K: garlic (30%); L: control; M: vinegar (50 mM); N: vinegar (75 mM); O: vinegar (100 mM); P: control; Q: sodium benzoate (50 mM); R: sodium benzoate (75 mM); S: sodium benzoate (100 mM); T- control.

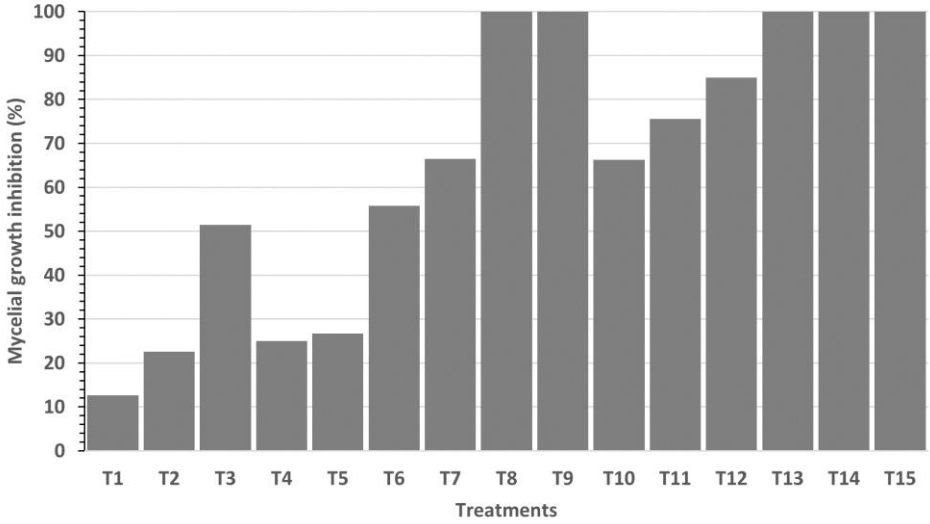

Fig. 5: Effect of aqueous plant extracts and food preservatives on mycelial growth inhibition of *FIESC* at 7 dpi. Here, T1: black cumin (10%); T2: black cumin (20%); T3: black cumin (30%); T4: turmeric (10%); T5: turmeric (20%); T6: turmeric (30%); T7: garlic (10%); T8: garlic (20%); T9: garlic (30%); T10: vinegar (50 mM); T11: vinegar (75 mM); T12: vinegar (100 mM); T13: sodium benzoate (50 mM); T14: sodium benzoate (75 mM); T15: sodium benzoate (100 mM).

Our results conform to the findings of Barman et al. (2015) who assessed the effect of crude garlic extracts against *Fusarium solani*, in which 100% mycelial inhibition was found due to 40% extract. Likewise, Braman et al. (2015) reported an antifungal activity of ethanolic extracts (1.0 mg/ml) of garlic bulbs and showed a significant activity for *Aspergillus* sp. *Penicillium* sp. and *Fusarium* sp. Besides, Shamsi and Chowdhury (2016) revealed that 20% ethanolic garlic concentrations completely inhibited the vegetative growth of *Fusarium moniliforme*. Barman et al. (2015) revealed that garlic extract (0.1%) showed 98.2% inhibition of mycelial growth of *Pestalotiopsis theae,* the causal organism of the grey blight of tea. Chemical analysis revealed that major volatile components in garlic were detected as allicin, ajoene and alliin (Goncagul and Ayaz 2010). Garlic extracts have also been shown to decrease the oxygen uptake of microbes, reduce the growth of pathogenic organisms, and inhibit the synthesis of lipids, proteins, and nucleic acids, and damage to membranes of microorganisms (Harris et al. 2001).

Effect of food preservatives against targeted fungus

In the present investigation, there was the upward trend of vegetative growth inhibition of the targeted fungus with increasing doses of vinegar. The utmost inhibition of mycelial growth (85%) of FIESC was measured due to 100mM of vinegar, followed by 75% and 68% inhibition by 75 mM and 50 mM, respectively (Fig. 4-5).

Importantly, sodium benzoate exhibited outstanding outcomes in which the lower dose of sodium benzoate (50 mM) was quite enough for the complete mycelial inhibition of FIESC (Fig. 4-5). Shi et al. (2016) reported the positive effects of vinegar against the mycelial growth of *Fusarium oxysporum* f. sp. *cucumerinum* and incidence of *Fusarium* wilt. Chuaboon et al. (2016) found that *Fusarium semitectum* was inhibited due to vinegar under *in vitro* conditions. Hassan et al. (2015) evaluated the efficacy of several organic acids, in

which vinegar (10%) showed the maximum inhibition of *Aspergillus flavus*, followed by *Penicillium purpurogenum*. Organic acids are known as safe compounds to preserve foods. Importantly, these acids act on the plasmic membrane of susceptible fungi by enhancing permeability and neutralizing its electrochemical potential (Hauka et al. 2005). In our study, complete mycelial inhibition of FIESC was seen due to sodium benzoate, which is supported by earlier findings. Alsudani (2017) showed that sodium benzoate had very high antifungal activity against several saprophytic fungi. Elsherbiny and El-Khateeb (2012) revealed that the mycelial growth of *Fusarium solani* and *Helminthosporium solani* was completely inhibited by sodium benzoate at the lowest concentration (0.1 M). The sporulation of pathogens was also strongly inhibited by sodium benzoate at all concentrations. Sodium benzoate was also the most effective compound in inhibiting spore germination (100%) for both fungi. Other lab studies showed that sodium benzoate (0.2 M) inhibited the mycelial growth of the economically important potato pathogens *H. solani* (Hervieux *et al.* 2002) and *Fusarium sambucinum* (Mecteau et al. 2002) completely. The various modes of action of weak acid preservatives are through membrane disruption (Palou et al. 2001), inhibition of essential metabolic functions, stresses on pH homeostasis, and the accumulation of toxic anions within the cell (Mitchell and Walters 2004). The principal mode of action of weak acid preservatives, however, is believed to be the passage of the un-dissociated compound through the plasma membrane; once inside the cell in a higher pH environment, the acid dissociates causing an accumulation of protons and anions, which cannot re-cross the plasma membrane (Droby et al. 2003).

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(Manuscript received on 30th November 2023 and revised on 9th January 2024)