



Molecular characterization and *in vitro* control measures of fruit rot disease of Sweet pepper

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ABSTRACT

Fruit rot disease of sweet pepper is one of the main fungal diseases causing huge economic losses to the grower. An experiment was conducted to find out the fungal pathogen associated with fruit rot disease of sweet pepper, obtained from experimental fields of Jahangirnagar University, Bangladesh. Fruit rot disease-causing fungus was isolated from infected fruits and identified using morphological characterization based on colony features, mycelia, conidia as well as molecular characterization based on internal transcribe spacer (ITS) region of the fungus. ITS sequence of our studied fungus MH368146.1 was genetically 99-100% similar to sequences of *Fusarium solani* in NCBI database. Typical fruit rot symptoms were reproduced by artificial inoculations of the isolated fungus. The mycelial growth of this fungus was evaluated on ten different solid culture media i.e., Potato Dextrose Agar, Yeast Extract Agar, Honey Peptone Agar, Hansen's Medium, Sabouraud's Glucose Agar, Kauffman's Agar, Potato Sucrose Agar, Richard's Agar and Carrot Agar. Fungus grew well on all tested solid culture media. Several bio-control agents and two commercial fungicides were evaluated against isolated fungus under *in vitro* condition, in which the highest percent inhibition of radial growth of the fungus was determined as 64.75% due to *Trichoderma reesei* isolate 2, and 60.63% by Tilt 250 EC (500 ppm) at 7 days post-incubation. Therefore, *T. reesei* was found as the most suitable to control the growth of *F. solani* under laboratory conditions. However, further pot and field trials needed to be confirmed the bio-control potential of it.

Keywords: *Fusarium solani*, Culture media, Bio-control agents, Fungicides, Bangladesh

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Introduction

Sweet pepper (*Capsicum annum*) belongs to the Solanaceae family, is one of the important vegetables in most European and North American countries. This crop is mostly grown in glasshouse conditions. Recently, this high-value crop has been introduced in Bangladesh, and farmers are growing in open field conditions. Several fungal diseases can cause serious economic losses on sweet pepper productivity such as seedling damping-off (*Pythium* spp. and *Rhizoctonia solani*), Pythium crown and root rot, Fusarium stem and fruit rot (*Fusarium solani*), anthracnose (*Colletotrichum* spp.), Cercospora leaf spot (*Cercospora capsici*), gray mold (*Botrytis cinerea*), grey leaf spot (*Stemphylium* spp.), southern blight (*Sclerotium rolfsii*), Verticillium wilt (*Verticillium* spp.), and powdery mildew (*Leveillula taurica*). Stem and fruit rot are damaging sweet pepper diseases caused by *Fusarium solani* (Mart.) Sacc., which usually caused around 5% fruit loss in Canada (Howard

et al., 1994). However, a severe outbreak of this disease resulted in a 50% yield loss in Ontario, Canada under greenhouse in 1990 (Jarvis *et al.*, 1994). Other species such as *Fusarium subglutinans* (Utkhede and Mathur, 2004), *Fusarium lactis*, *Fusarium proliferatum* and *Fusarium oxysporum* (Yang *et al.*, 2009) also known to cause fruit rot of sweet pepper. Diseases causing fungal spores germinate on the young seeds or internal parts of the fruits at early stages of infections, and sunken lesions appear on the fruit surfaces at later stages (Poucke *et al.*, 2012). Even, this symptom appears at fruit development and post-harvest stages, the initial fungal infection takes place during flower stages via deposition of fungal spores on stigma, followed by fungal growth through style into the ovary (Yang *et al.*, 2010). Organic and ecological harmless substitutes, for example, bio-control agents, natural plant metabolites, and cultural techniques are being investigated for conceivable



use in integrated disease management platforms. Therefore, considering all of the above facts, an attempt has to be made to isolate and identify the fungus that causes fruit rot of sweet pepper, evaluate the effect of culture media on the mycelial growth of the fungus, and assess the efficacy of bio-control agents and fungicides against the fungus.

Materials and Methods

Isolation, identification, and pathogenicity

Tissue planting methods were used to isolate the fungal pathogen from infected sweet pepper fruits. For morphological identification, standard manuals were used- Dematiaceous Hypomycetes by Ellis (1971). The fungus genomic DNA samples were extracted using Maxwell Cell Kit (AS1030, Promega, USA). The primer ITS4 (5-TCCCTCCGCTTATTGATATGC-3) and ITS5 (5-GGAAGTAAAAGTCGTAACAAGG-3) were used to amplify target region of the fungus (White *et al.*, 1990). The PCR was performed in a 25 µl reaction mixture, consisting of 12.5 µl GoTaq G2 Hot Start Green Master Mix (dNTPs, Buffer, MgCl₂, Taq Polymerase; Promega 2X, Promega, Madison, USA), 2.5 µl of each primer (10 µM), 5 µl DNA template (20 ng/µl), and 2.5 µl water. The PCR reaction was conducted with the activation of Taq polymerase at 94°C for 1 minute, 35 cycles of 94°C for 30 Sec, 55°C for 30 Sec, 72°C for 5 minutes, and termination with a 10-minute step at 72°C (Sikder *et al.*, 2019). Maxwell® 16 DNA Purification Kits (Promega, USA) was used to purify the amplification products. The purified PCR product of approximately 650 bp was sequenced in First BASE Laboratories Sdn Bhd (Malaysia). Sequencing data was blast searched and compared for percent homology of rDNA sequence with similar DNA sequences retrieved from NCBI Genbank database. The phylogenetic analysis was conducted using the multiple sequence alignment tools and a maximum likelihood tree was generated using MEGA 6 software (Tamura *et al.*, 2013; Sikder *et al.*, 2020).

To test of pathogenicity, three healthy seedlings of sweet pepper were transplanted in each earthen pot (10-inch diameter) containing sterilized soil and allowed to grow for 25 days in net house providing necessary water and nutrients. The conidial suspension (10⁴/ml) of *F. solani* was prepared from seven days old fungal culture and sprayed on the healthy potted plant. Control plants received only sterilized water without fungal inoculum. Symptoms produced on artificially inoculated plants were recorded and compared with those observed on naturally infected plants. The fungus was re-isolated from the inoculated plants of sweet pepper on PDA medium to fulfill Koch's postulate.

Effect of solid culture media on mycelial growth of F. solani

Nine different culture media i.e. Potato Dextrose agar, Yeast Extract Agar, Honey Peptone Agar, Hansen's Medium, Sabouraud's Glucose Agar, Kauffman's Agar, Potato Sucrose Agar, Richard's Agar and Carrot Agar were prepared to investigate the mycelial growth of the fungus (Sultana *et al.*, 2020). Radial growth of mycelia on each Petri dish was measured at 3 and 7 days post-incubation with 3 replicates each. Data on mycelial growth characteristics were checked for normality and homogeneity of variance; found to be normal and analyzed using one-way ANOVA with Duncan's Post-Hoc test in SPSS.

Efficacy of bio-control agents and fungicides against F. solani

The bio-control agents- *Bacillus subtilis*, *Pseudomonas fluorescens*, *Trichoderma harzianum* and two isolates of *Trichoderma reesei* were evaluated against mycelial growth of the fungus using dual culture technique (Bhadra *et al.*, 2014). The effect of fungicides, namely Tilt 250 EC (Propiconazole) and Amistar Top 325 SC (Azoxystrobin + Difenconazole) on the radial growth of the fungus was determined on PDA medium using the food poison technique (Rahman *et al.*, 2015). A requisite quantity of fungicides was added to the medium with a concentration of 250 ppm, 500 ppm, and 750 ppm. Percentage inhibition of the pathogen was calculated (Shamoli *et al.*, 2016) using the formula: $I = (C-T/C) \times 100$ Here, I = percentage of mycelium growth inhibition, C = growth of mycelium in control, T = growth of mycelium in treatment.

Results and Discussion

Identification and pathogenicity of the fungal pathogen

Symptoms of the disease on sweet pepper caused by the fungus have shown in Figure 1A. The sunken lesion was appeared on developing sweet pepper fruits. The colony was more or less circular or slightly irregular depending on culture media, with abundant milky white-colored conidial masses. The mycelia were profusely developed white floccose colony on PDA medium (Figure 1B). The hyphae of the fungus were hyaline and septate. The conidia were born on distinct, well-developed, hyaline conidiophores. Conidiophores were unbranched and monophialides. Microconidia were oval-ellipsoidal shaped with no septation while macroconidia were sickle shaped, hyaline, with 2-3 septation (Figure 1C). Based on morphological features, it was identified as *Fusarium* sp. It was quite difficult to identify the fungus at species rank without molecular characterization.

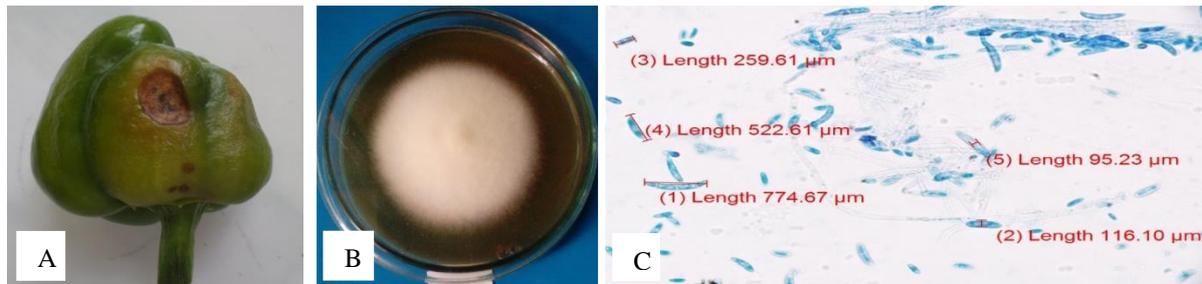


Figure 1. A. Disease symptoms on sweet pepper fruits; B. fungal pure culture on PDA medium; C. Micro-photograph of the isolated fungus.

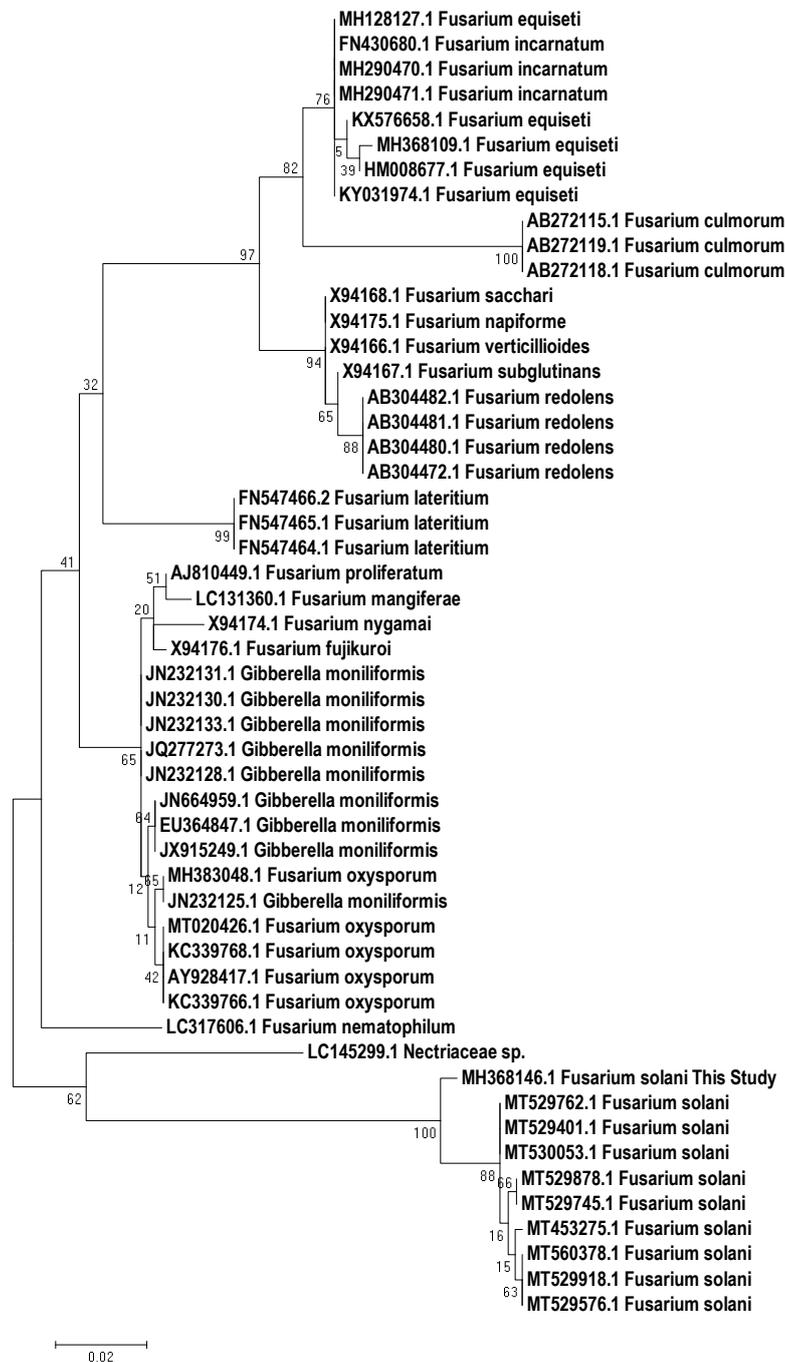


Figure 2. The maximum likelihood tree represents the relationship between *F. solani* and other species of the genus *Fusarium*. Our fungal pathogen is marked as this study.

After submission of nucleotide sequence of the studied fungus, accession number MH368146.1 was received and identified as *Fusarium solani*. In the maximum likelihood tree, our studied fungal pathogen *F. solani* (MH368146) was formed a completely separate cluster with *F. solani* group with boot-strap value of 100 (Figure 2). [Ahmmmed *et al.* \(2020\)](#) showed that characterization by molecular techniques using polymerase chain reaction to amplify the ITS allows identifying organisms that cannot be distinguished morphologically.

The association of *F. solani* with fruit rot of sweet pepper was further confirmed by a pathogenicity test. The healthy susceptible cultivar of sweet pepper was grown in pots that were inoculated with the respective fungal pathogen under controlled conditions. The typical fruit rot symptoms were observed. The re-isolation of the fungal pathogen proved the pathogenic nature of a particular fungus *F. solani*.

Effect of culture media on the mycelial growth of *F. solani*

The effect of different solid media on the mycelial growth of *F. solani* was evaluated and results revealed that most of the culture media supported the vegetative growth of the fungus

(Figure 3 and 4). The highest mycelial growth of *F. solani* was recorded on Honey Peptone Agar, Hansen's Medium, Kauffman's Agar, Potato Sucrose Agar, Richard's Agar, and Carrot Agar. A number of reports on the mycelial growth of *Fusarium* spp. have been found under different fungal culture media but there were no previous findings available regarding the effect of honey peptone agar on mycelial growth of *F. solani*. The profuse growth of the fungus was noticed probably due to the presence of high sugar content in honey (38% fructose, 31% glucose, 5% dextrin, and 1.5-3% sucrose). [Gupta *et al.* \(2010\)](#) obtained the maximum mycelial growth on potato dextrose agar as semi-solid media for *F. oxysporum* f.sp. *psidii* and *F. solani*. [Chittem and Kulkarni \(2008\)](#) studied the growth characters of *F. oxysporum* f.sp. *dianthi* studied on different solid media indicated that, potato dextrose agar, Richards's agar, Czapek's Dox agar, and Oat meal agar supported the maximum growth of the fungal colony. In another study, the V8 and malt extract agar culture media exhibited the maximum diameter of the fungal (*F. oxysporum* and *F. solani*) colonies, while the malt extract agar and potato sucrose agar media contributed to the highest sporulation of *Fusarium* spp ([Mezzomo *et al.*, 2018](#)).

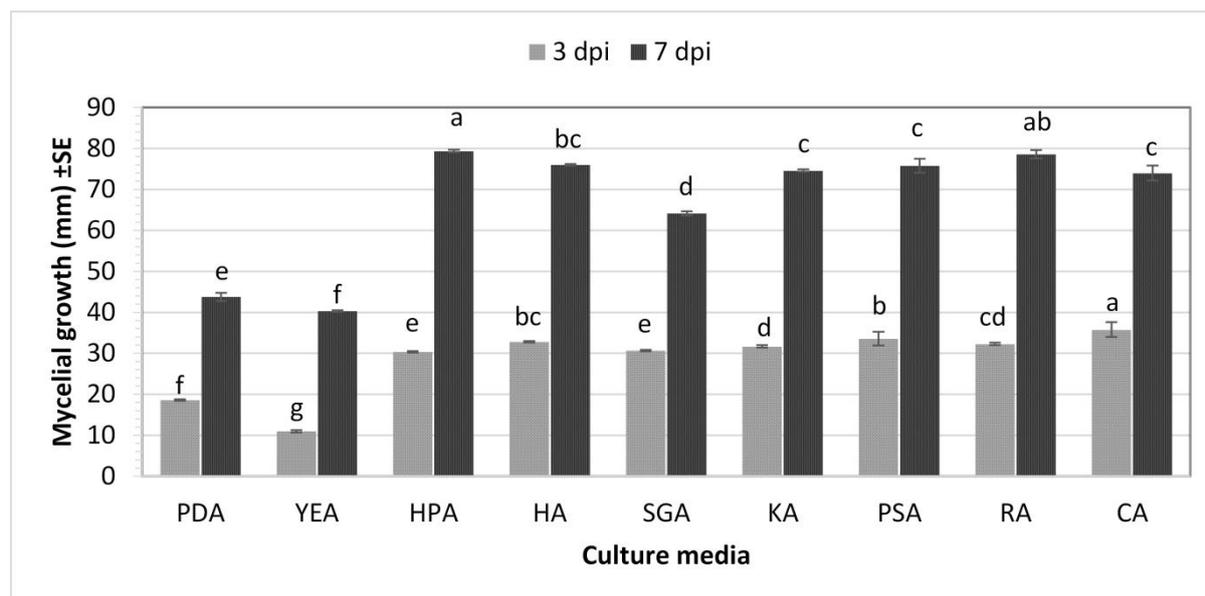


Figure 3. Effect of fungal culture media on the mycelial growth of *F. solani* at 3 and 7 days post incubation (dpi), respectively. PDA: Potato dextrose agar; YEA: Yeast Extract Agar; HPA: Honey Peptone Agar; HA: Hansen's Medium; SGA: Sabouraud's Glucose Agar; KA: Kauffman's Agar; PSA: Potato Sucrose Agar; RA: Richard's Agar; CA: Carrot Agar. The value represents as mean \pm standard error (SE) of three replications. Means followed by common letter (s) do not differ significantly at 5% level by DMRT.



Figure 4. Photograph showing an effect of fungal culture media on the mycelial growth of *F. solani* at 7 dpi. A: Potato dextrose agar; B: Yeast Extract Agar; C: Honey Peptone Agar; D: Hansen's Medium; E: Sabouraud's Glucose Agar; F: Kauffman's Agar; G: Potato Sucrose Agar; H: Richard's Agar; I: Carrot Agar.

***In vitro* efficacy of biological organisms against the fungal pathogen**

The antagonistic effect of fungi *Trichoderma reesei* isolate 1, *Trichoderma reesei* isolate 2, *T. harzianum* and rhizospheric soil bacteria *Bacillus subtilis* and *Pseudomonas fluorescens* on the inhibition of radial mycelial growth of *F. solani* were observed at 3 dpi and 7 dpi at room temperature (Figure 5 and 6). The highest percent inhibition of radial growth of the fungal pathogen was calculated as 25.09% due to *T. reesei* isolate 2 and the least of 0.0% by *P. fluorescens* at 3 dpi. Similar trends of mycelia growth inhibition of *F. solani* were recorded at 7 dpi, in which 64.75% mycelia inhibition was recorded due to *T. reesei* isolate 2, which was close to *Bacillus subtilis* (62.83%), *T. reesei* isolate 1 (61.5%), and *T. harzianum* (61.5%). The lowest of 7.3 % inhibition was found due to *P. fluorescens*. Our results are supported by [Bhadra *et al.* \(2016\)](#), who reported that *Trichoderma* based bio-control agents were successful in restricting the vegetative growth of *F. solani*,

causing wilt of brinjal. Likewise, [Khan *et al.*, \(2014\)](#) investigated the wilt disease complex of chickpea caused by *Fusarium oxysporum* and reported the reduction of disease incidence due to *Trichoderma*-based bio-control formulation. Besides, [Abdulkareem *et al.* \(2014\)](#) reported that the colony diameter of *Fusarium graminearum* was significantly reduced due to the use of rhizospheric bacteria. [Ramamoorthy and Samiyappan \(2001\)](#) cited that *P. fluorescens* effectively inhibited the mycelial growth of *C. capsici* under *in vitro* and decreased the fruit rot incidence in chilli under greenhouse conditions. However, we could not find any significant performance of *P. fluorescens* against *F. solani* under *in vitro* conditions; these could be due to the variation of isolates used in the present study. Our study revealed an antagonistic effect of bio-control agents (*T. reesei*, *T. harzianum* and rhizospheric soil bacteria *B. subtilis*) against *F. solani* under lab bioassay. However, a pot and field trial is needed to confirm the efficacy of these organisms.

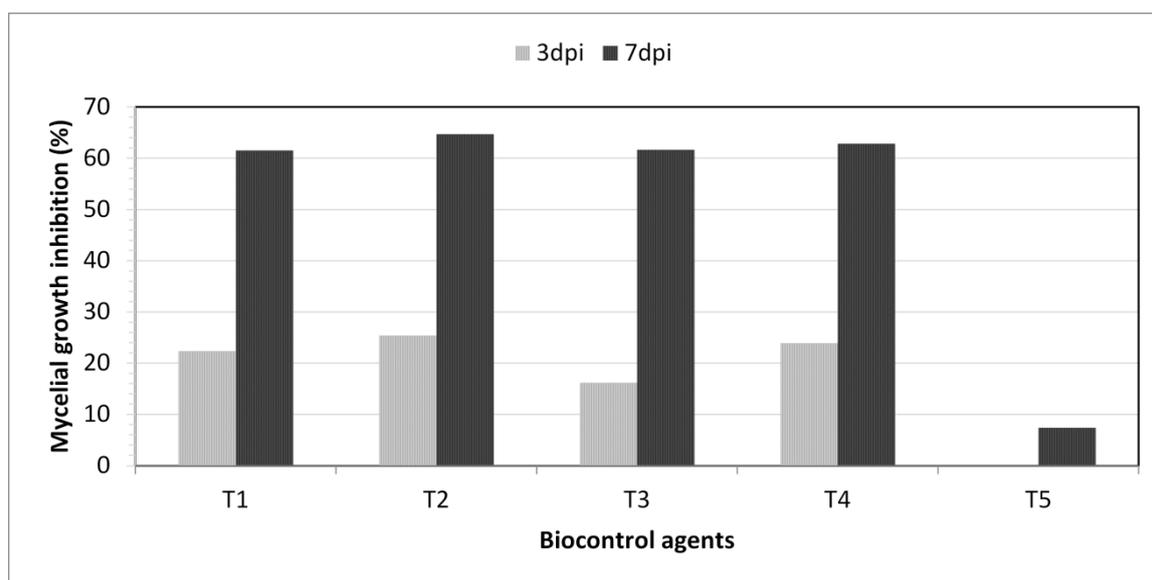


Figure 5. Antagonistic effect of bio-control agents on the mycelial growth inhibition (%) of *F. solani* at 3 dpi and 7 dpi, respectively. Here, T1: *Trichoderma reesei* isolate-1, T2: *Trichoderma reesei* isolate-2, T3: *Trichoderma harzianum*, T4: *Bacillus subtilis*, T5: *Pseudomonas fluorescens*.

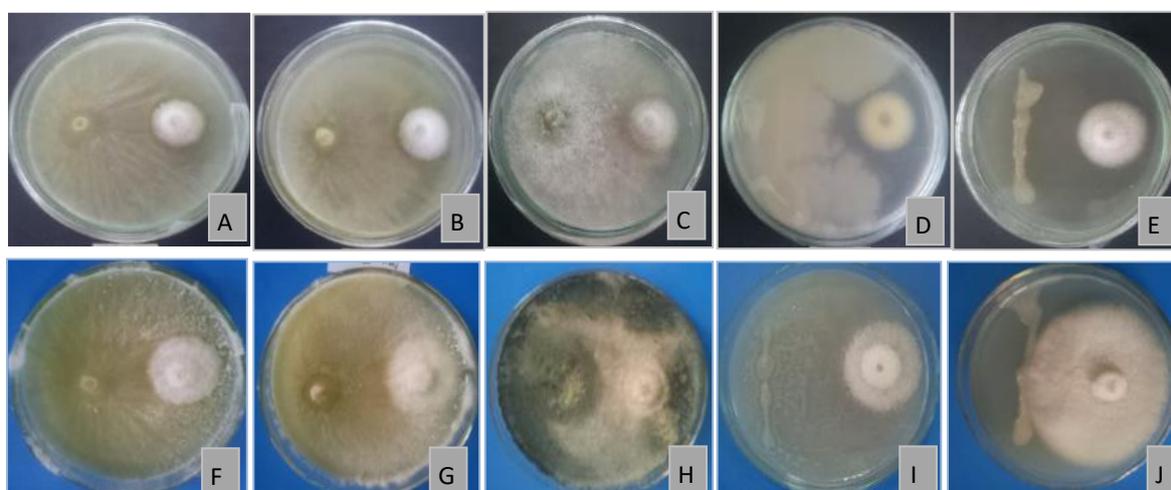


Figure 6. Photograph showing an antagonistic effect of bio-control agents on the mycelial growth inhibition of *F. solani* at 3 dpi (A-E) and 7 dpi (F-J). *T. reesei* isolate 1 vs. *F. solani* (A, F); *T. reesei* isolate 2 vs. *F. solani* (B, G); *T. harzianum* vs. *F. solani* (C, H); *B. subtilis* vs. *F. solani* (D, I); *P. fluorescens* vs. *F. solani* (E, J).

Efficacy of fungicides against the fungal pathogen

The efficacy of fungicides Tilt 250 EC (propiconazole) and Amistar Top 325 SC (Azoxystrobin + Difenconazole) on the mycelium growth of *F. solani* were evaluated (Figure 7 and 8). In general, there was an increasing mycelial inhibition found with increasing doses of fungicides. Similar trends of mycelial inhibition both at 3 and 7 dpi were detected; around 60% of mycelial inhibition was recorded by both 500 ppm and 750 ppm of Tilt 250 EC. Amistar Top 325 SC had more than 50% mycelial growth inhibition due to 750 ppm concentration both at 3 and 7 dpi. In the study, Tilt 250 EC was able to restrict 60% mycelial

growth of *F. solani*, which is supported by Chowdhury *et al.* (2015) who reported that fungicides Tall 25 EC (propiconazole) at 200, 300, 400, and 500 ppm were effective against *Fusarium moniliforme* under *in vitro* condition. Likewise, Tilt fungicide significantly inhibited the mycelial growth of *F. oxysporum* f.sp. *cubense* *in vitro* conditions and as root drench reduced the Fusarium wilt of banana incidence by 75% in the greenhouse condition (Nel *et al.*, 2007). Similarly, Tilt was found to be effective against *F. oxysporum* causing tobacco wilt under *in vitro* (Sumana *et al.*, 2011) and field conditions (Sumana *et al.*, 2012). Therefore, Tilt 250 EC with increasing doses might be used to manage the fruit rot disease of sweet pepper.

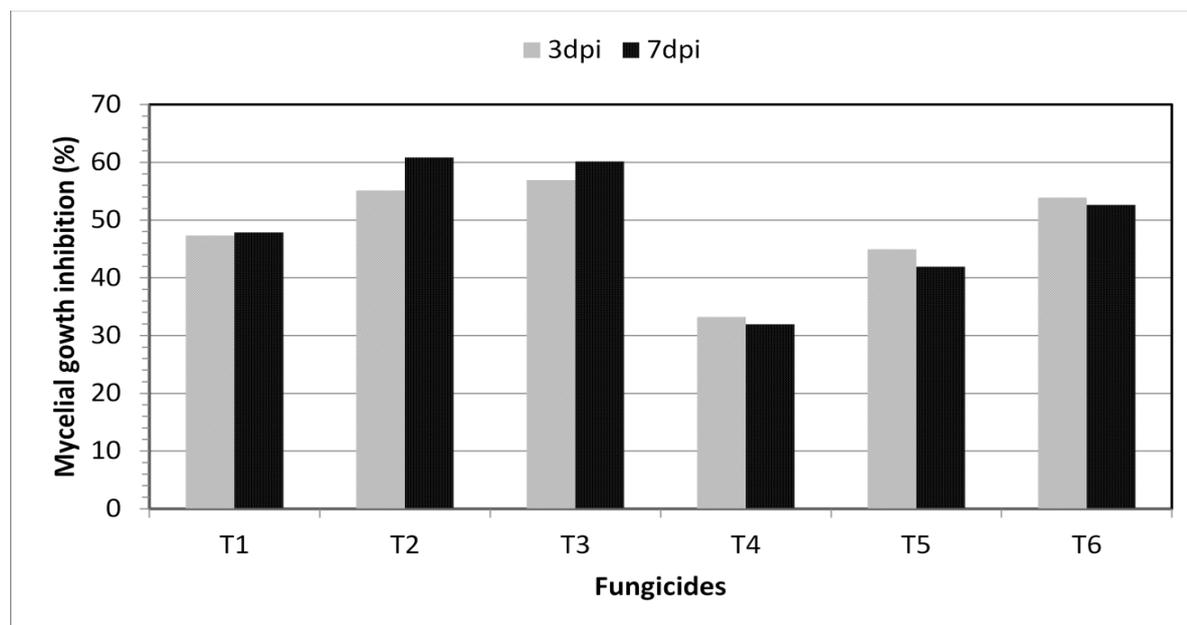


Figure 7. Effect of fungicides on the mycelial growth inhibition (%) of *F. solani* at 3 and 7 dpi, respectively. Here, T1: Tilt 250 EC (250 ppm), T2: Tilt 250 EC (500 ppm), T3: Tilt 250 EC (750 ppm), T4: Amistar Top 325 SC (250 ppm), T5: Amistar Top 325 SC (500 ppm), T6: Amistar Top 325 SC (750 ppm).

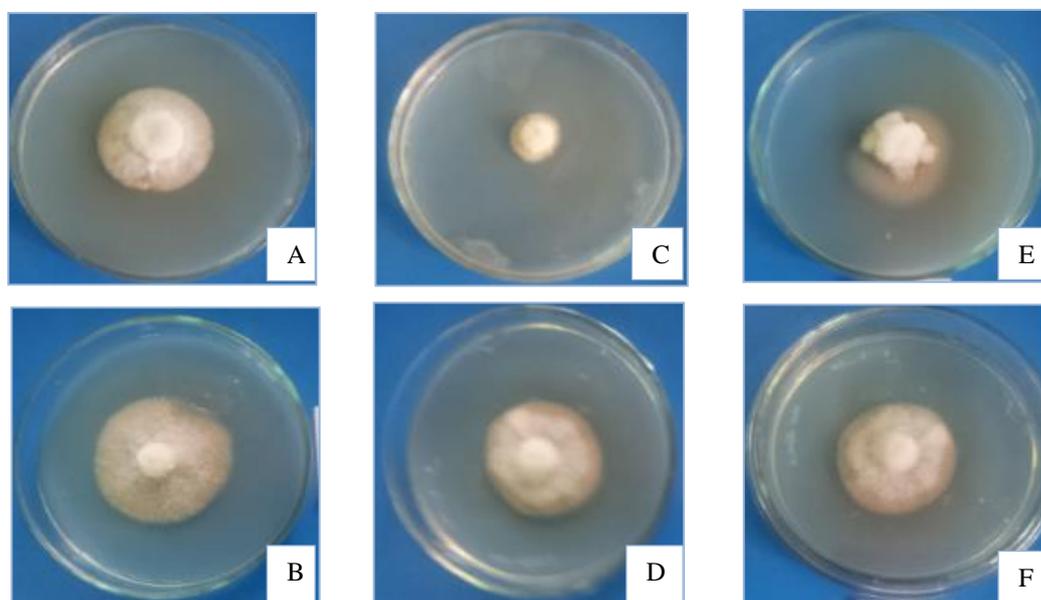


Figure 8. Photograph showing an effect of fungicides on mycelial growth inhibition of *F. solani* at 7 dpi. A: Tilt 250 EC (250 ppm); B: Amistar Top 325 SC (250 ppm); C: Tilt 250 EC (500 ppm); D: Amistar Top 325 SC (500 ppm); E: Tilt 250 EC (750 ppm); F: Amistar Top 325 SC (750 ppm).

Conclusion

Fusarium solani causing fruit rot disease of sweet pepper is responsible for reducing the quantity and quality of the product at pre and post-harvest period. Morphology and molecular characterization have confirmed the identity of fungus; further pathogenicity tests confirmed the pathogenic nature of it. The phylogenetic tree was generated with retrieved ITS sequences analysis by maximum likelihood to demonstrate the position of *F. solani*. Subsequently, the effects of

different culture media on fungal nourishment were evaluated and found honey peptone agar medium as the most suitable for mycelial growth of the fungus. The study also explored *in vitro* efficacy of several bio-control agents and two commercial fungicides against *F. solani*. This study could be the basis for field trials to find out the efficacy of these tested bio-control agents and fungicides to manage fruit rot disease of sweet pepper.

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