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FIRST REPORT OF STRAWBERRY LEAF BLIGHT CAUSED BY *FUSARIUM FUJIKUROI* SPECIES COMPLEX IN BANGLADESH

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Abstract

Strawberry leaf blight is one of the devastating fungal diseases, responsible for reducing the quality of strawberry production worldwide. The present experiment was conducted to determine the pathogenic fungal association with strawberry leaves obtained from the experimental site of Botanical Garden, Jahangirnagar University, Bangladesh. A fungal pathogen causing leaf blight disease of the strawberry plant was detected as *Fusarium fujikuroi* species complex through classical and molecular approaches. Molecular characterization showed that nucleotide sequences of the internal transcribed region of the studied fungus (MH368119.1) were genetically identical with *Fusarium fujikuroi* species complex. Different culture media, pH, light regimes, temperatures were assayed to know the growth pattern of the identified pathogenic fungus. The optimum vegetative growth of *Fusarium fujikuroi* species complex was recorded on Potato Dextrose Agar, Potato Sucrose Agar, and Honey Peptone Agar media, at 25°C temperature, pH seven and under complete dark conditions. Among the three antagonistic fungi for the assessment as a biocontrol agent, *Trichoderma asperellum* was comparative effective against *Fusarium fujikuroi* species complex under *in vitro* conditions. Among systemic chemical fungicides, the highest mycelial inhibition (89.42%) of the isolated fungus was found by Tilt 250 EC (500 ppm). The leaf blight of strawberry caused by *Fusarium fujikuroi* species complex is the new record in Bangladesh.

***Key words*:** Antagonist, fungal biology, fungicides, ITS, leaf blight disease

Introduction

Strawberry (*Fragaria* × *ananassa* Duch.) is a stoloniferous and perennial herb under Rosaceae. There are 500 commercial cultivars of strawberries worldwide (Rahman et al. 2015a). The plant's growth is generally influenced by chilling, whereas critical day length (short day) and temperature conditions play an important role in flowering. The winter season is suitable for strawberry production in Bangladesh; planting time is October and harvesting from January to April (Biswas et al. 2008). Several strawberry varieties, namely- BARI strawberry-1, BARI strawberry-2, BARI strawberry-3, and BADC strawberry, were released by Bangladesh Agricultural Research Institute and Bangladesh Agricultural Development Corporation (Hossain et al. 2013, Rahman et al. 2015a).

Several strawberry diseases were reported in Bangladesh, such as strawberry anthracnose disease causing by *Colletotrichum gloesporioides* (Akhter et al. 2009), strawberry leaf spot disease causing by *Neopestalotiopsis chrysea* (Sultana et al. 2022), strawberry crown rot disease causing by *Pestalotiopsis* sp. (Ara et al. 2017); *Colletotrichum siamense* (Gupta et al. 2018), Fusarium wilt of strawberry causing by

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Fusarium oxysporum (Surovy et al. 2018). However, no report was found on the association of *Fusarium fujikuroi* species complex with strawberry leaf blight disease. Correct diagnosis of the pathogenic fungi is imperative to design suitable management strategies. Besides, identification of fungi based on morphological features sometimes lead to erroneous conclusions. Therefore, molecular identification of fungi using DNA barcoding (ITS region) has become an essential part of fungal ecology research. The current study was conducted to correct the diagnosis of fungal strawberry pathogen, investigate growth factors (pH, temperature, light and culture media) of the isolated fungus, and evaluate the efficacy of antagonistic fungi and different systemic and contact chemical fungicides against the isolated pathogenic fungus under *in vitro* conditions.

Materials and Methods

Sample collection, isolation, identification, and pathogenicity

Leaf blight disease-infected strawberry leaves were collected from the experimental sites of the Botanical Garden of Jahangirnagar University, Savar, Dhaka, Bangladesh. Leaves samples were surface sterilized by 5% sodium hypochloride, washed with distilled water, air dried, and placed on PDA medium. Subculture of the isolated fungus was carried out, and pure culture was stored at -4°C freezer until further uses. The isolated fungal pathogen was identified based on colony morphology, mycelium, and conidia features.

The fungal genomic DNA was extracted using commercial kits (Promega Wizard DNA Extraction Kit, USA). Two universal primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') was used for PCR amplification (Sikder et al. 2019). The PCR reaction was performed with activation of *Taq* polymerase at 94°C for 5 min, followed by 35X (94°C for 30 sec, 57°C for 30 sec, and 72°C for 5 min.) and termination at 72°C for 10 minutes (Ahmmed et al. 2020). The amplicons were purified (Maxwell® 16 DNA Purification Kits, Promega, USA) and sent for sequencing (FIRST BASE Laboratories, Sdn Bhd, Malaysia). Sequencing data compared through BLAST search in NCBI Genbank database. The multiple sequence alignment tools were used to generate a phylogenetic (Neighbor-joining) tree using MEGA 6 software. The detached leaf inoculation technique was applied to test pathogenicity (Aktar and Shamsi 2014).

Effect of culture media, temperature, pH, and light of the pathogenic fungus

The growth pattern of the isolated fungus was evaluated on different soil fungal culture media (potato dextrose agar, PDA), (potato sucrose agar, PSA), (carrot agar, CA), (Richard agar, RA), (Honey peptone agar, HPA), and (Honey agar, HA) as described by Mallik et al. (2021). The response of different temperature conditions (15, 20, 25, 30 and 35°C) was evaluated for the vegetative growth of the isolated fungus as the method mentioned by Billah et al. (2021). The five different pH conditions (5, 6, 7, 8 and 9) were used to assess the mycelial growth of the fungus (Sikder et al. 2020). The impact of three different light conditions (24 h dark, 24 h light, and the alternate cycle of 12 h light-dark) on the vegetative growth of the isolated fungus was evaluated (Ahmmed et al. 2020). The vegetative growth of the fungus was recorded at seven days post-inoculation (dpi).

In vitro mycelial growth inhibition of pathogenic fungus

The vegetation growth inhibition (%) of the isolated fungus was determined using duel culture techniques, where antagonist fungi, namely-*Trichoderma reesei*, *Trichoderma harzianum*, and *Trichoderma asperellum* were used (Bhadra et al. 2014). Chemical fungicides-Tilt 250 EC and Amistar Top 325 SC @ 100 ppm, 250 ppm, 500 ppm; Ridomil gold MZ 68WP @ 250 ppm, 500 ppm, and 750 ppm were used to assess their efficacy against the isolated fungus under laboratory conditions, in which poison food techniques was

employed (Rahman et al. 2015b). The PDA plates contained without any fungicides served as control. Data was recorded at seven dpi. The percent mycelial growth inhibition was determined using standard formula (Bhadra et al. 2016) and analyzed using one-way ANOVA with Duncan's Post-Hoc test in SPSS-20.

Results and Discussion

Isolation and identification of the fungal pathogen

Strawberry leaf blight infections were initiated as circular reddish-purple spots on the leaf. These spots turned into V-shaped dark brown lesions with an outer yellow zone (Fig. 1A). Lesions also formed in the main leaf veins with the progression of time. Finally, the whole leaflet turned brownish at a later stage. This disease severely attacked old leaves. The mycelium of the fungus was whitish to light pink (Fig. 1B). The conidiophores were unbranched and monophialides. The hypha was hyaline and septate. The microconidia were single celled, ovoid to oblong, dumbbell shaped (Fig. 1C). Morphological features suggested the fungal pathogen was *Fusarium* sp. Furthermore, the nucleotide sequencing of ITS region of the fungus (MH368119.1) showed above 99% sequence similarity with *Fusarium fujikuroi* species complex (MF281281.2, MN905474.1, MW827608.1), thus identified as *Fusarium fujikuroi* species complex. Besides, currently studied fungal pathogen formed a completely separate cluster with other *Fusarium fujikuroi* species complex (99% bootstrap value) in the phylogenetic tree (Fig. 2). Moreover, leaf blight symptoms appeared after artificial inoculation with the isolated fungus.

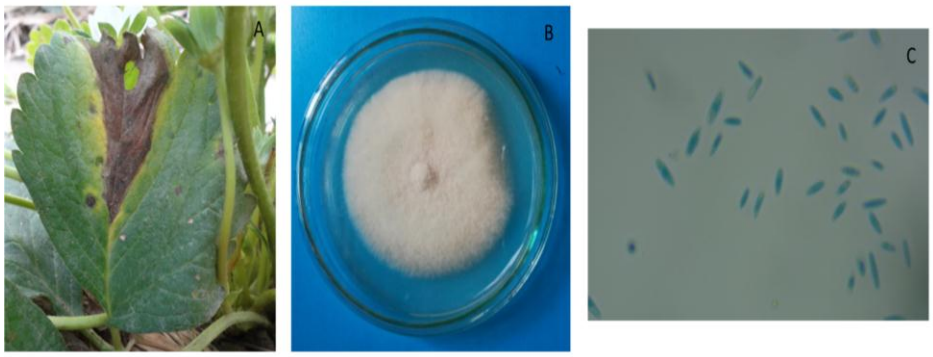

Fig. 1: A) Leaf blight symptoms on strawberry; B) fungal colony on PDA medium, and C) microscopic views of microconidia of *Fusarium fujikuroi* species complex (10 × 40X).

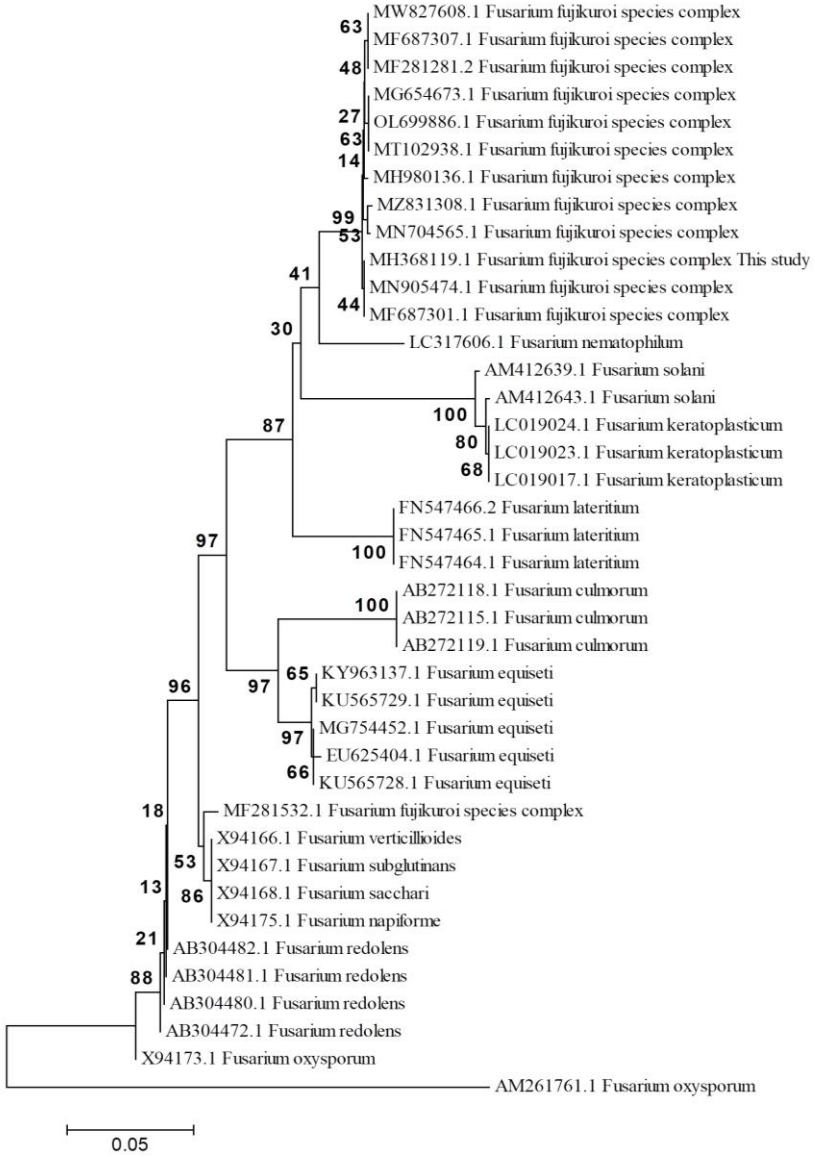

Fig. 2: Neibour joining tree of the studied organisms with bootstrap value. Our fungal pathogen is marked as this study.

Growth characteristics of the fungus

The fungus formed a fluffy whitish color colony with irregular margin on PDA, PSA, CA, HPA, and RA media; brownish in center and whitish edges on HA medium. Results revealed a statistical difference between the fungal culture media to support the mycelial growth of the isolated fungus (Fig. 3A). The fungus *F. fujikuroi* species complex preferred to grow all of the fungal culture media (approximate 90 mm) except HA medium (Fig. 3A). Mycelial growth was found only 50.67 mm on HA medium at seven dpi. Likewise, Fovo et al.

(2017) worked on several fungal culture media where the PDA was found most preferable for vegetative growth of *Fusarium oxysporum*. Similarly, Gupta et al. (2010) recorded the utmost mycelial growth *Fusarium oxysporum* f.sp. *psidii* and *Fusarium solani* on PDA at 7 dpi. Zope et al. (2012) concluded that PDA, MEA, and rice meal agar media were suitable for the radial growth of *Fusarium oxysporum* fsp. *ciceri*. Chittem and Kulkarni (2008) showed that *Fusarium oxysporum* fsp. *dianthi* preferred to grow on PDA, RA, Czapek's Dox, and oat meal agar. Pradeep et al. (2013) studied several solid and liquid media in which *Fusarium moniliforme* KUMBF1201 isolated from paddy field soil grew well on PDA and potato dextrose broth media.

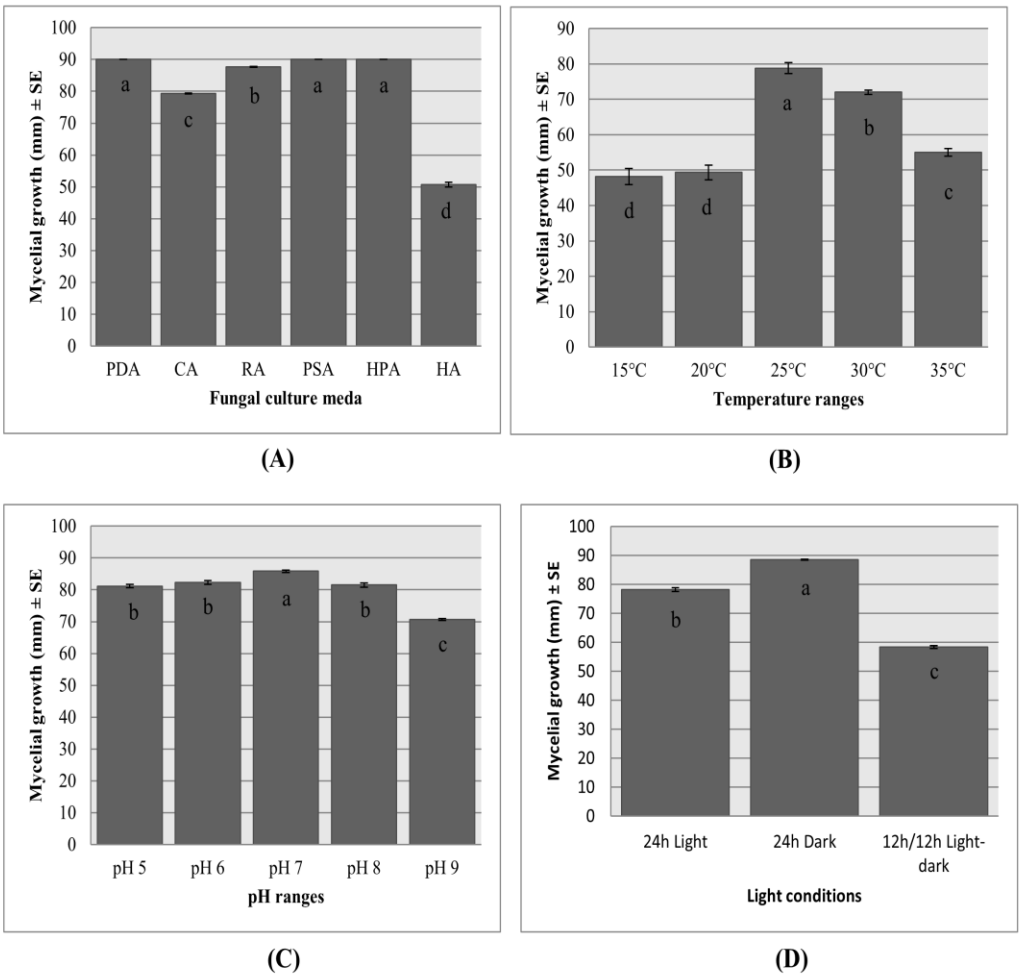

Fig. 3: Effect of culture media, temperature, pH and light on vegetative growth of *Fusarium fujikuroi* species complex at 7 dpi. Data represents mean ± standard error (SE) of six replications. PDA- potato dextrose agar, CA- carrot agar, RA- Richard's agar, PSA- potato sucrose agar, HPA- Honey peptone agar and HA- Hansen's agar.

Temperature is an important environmental factor that plays a regulatory effect on fungal cell metabolism, growth, and development. There were gradual trends of increasing mycelial growth with temperature rise. The higher peak of mycelial growth (78.83 mm) of *F. fujikuroi* species complex was noted at 25°C temperature, followed by 72 mm at 72°C and the lowest growth (48.17 mm) at 15°C temperature (Fig. 3B). Present findings conform to results of earlier researchers. Several *Fusarium* species preferred to grow at 25°C temperature conditions, such as *Fusarium oxysporum* fsp. *radicis-lycopersici* (Hibar et al. 2006), *Fusarium oxysporum* f. sp. *ciceri* (Farooq et al. 2005), *Fusarium oxysporum* f. sp. *lycopersici* (Kumar et al. 2011) at 25°C temperature. However, Khan et al. (2011) cited the optimum temperature for mycelial growth of *Fusarium oxysporum* f. sp. *ciceri* at 30°C. Likewise, Sahoo et al. (2015) cited that the proper growth vegetative growth of *Fusarium oxysporum* was also observed at 30°C temperature.

The variation in pH level of PDA media correlates with the mycelial growth of almost all of the fungus. The suitable pH of fungal culture media for the best growth and development of *F. fujikuroi* species complex was determined. The fungus showed a gradual increase of mycelial growth until neutral pH in which the maximum growth (85.83 mm) was recorded; then low growth (70.67 mm) was observed at pH 9.0 (Fig. 3C). Previous results support present finding. Mallik et al. (2021) isolated *Fusarium solani* from capsicum and found neutral pH as most suitable for mycelial growth. Moreover, the maximum mycelial growth of *Fusarium oxysporum* f.sp. *ciceri* (Farooq et al. 2005) and *Fusarium oxysporum* f.sp. *ciceri* (Khan et al. 2011) was also obtained at pH 7.

Results revealed that the isolated fungus showed the highest mycelial growth under complete dark conditions, followed by complete light conditions (Fig. 3D). The most nominal growth was observed in alternate 12 hours light/dark conditions (Fig. 3D). Our results are also supported by Mohsen et al. (2016) who citing the vegetative growth of several species of *Fusarium* (*Fusarium solani*, *Fusarium oxysporum*, *Fusarium sacchari*, *Fusarium proliferatum*, and *Fusarium globosum*) were significantly higher under continuous dark than continuous light conditions. However, Somu and Thammaiah (2015) obtained the maximum mycelial growth of *Fusarium oxysporum* f. sp. *cubense* at alternate cycles of light and darkness. In other studies, Sahoo et al. (2015) found alternate cycle of 12 hours of light and darkness was highly favorable for the growth of *Fusarium oxysporum*. Similar findings were also reported on the mycelial growth of *Fusarium oxysporum* fsp. *glycines* (Gheorghe et al. 2015) and *Fusarium oxysporum* f sp. *lini* (Pal et al. 2019).

Efficacy of antagonistic fungi and fungicides on the fungal pathogen

Results showed that the maximum mycelial growth inhibition of the fungus was recorded as 61.25% by *T. asperellum*, followed by 55.75% by *T. reesei*. In comparison, the lowest of 45.50% was found due to *T. harzianum* (Fig. 4). In present study, above 60% mycelial inhibition of *F. fujikuroi* was found due to fungal antagonists-*Trichoderma asperellum*, which is strongly supported by Ahmmed et al. (2021) also reported around 60% vegetative growth inhibition of *Fusarium oxysporum* due to *T. asperellum* under laboratory conditions. In another lab bioassay, Mallik et al. (2021) obtained the highest inhibition (64.75%) of radial growth of *Fusarium solani* by *T. reesei* followed by *T. harzianum*. Besides, Bhadhra et al. (2016) found promising effect against *Fusarium solani* f.sp. *melongenae* due to *Trichoderma* spp. under *in vitro* and *in vivo* conditions.

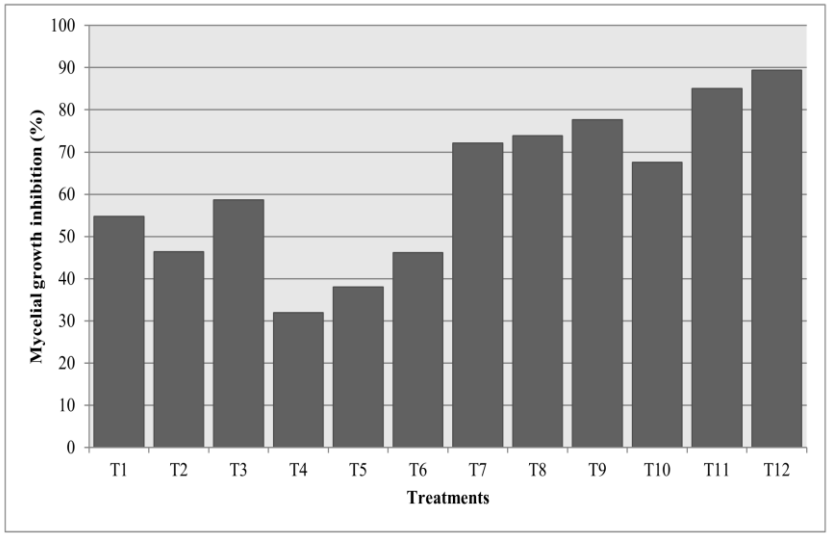

Fig. 4: Effect of biocontrol agents and chemical fungicides on the mycelial growth inhibition (%) of *Fusarium fujikuroi* species complex at 7 dpi. T1: *T. reesei*, T2: *T. harzianum*, T3: *T. asperellum*, T4: Ridomil Gold (250 ppm), T5: Ridomil Gold (500 ppm), T6: Ridomil Gold (750 ppm), T7: Amistar top (100 ppm), T8: Amistar top (250 ppm), T9: Amistar top (500 ppm), T10: Tilt 250 EC (100 ppm), T11: Tilt 250 EC (250 ppm), T12: Tilt 250 EC (500 ppm). Data represents as percentage value of six replications.

In our study, systemic chemical fungicides showed promising results regarding mycelial inhibition of fungal pathogen (Fig. 4). The mycelial growth inhibition of *F. fujikuroi* was enhanced with increasing doses of fungicides. The maximum mycelial growth inhibition (77.62%) of the fungus was found at 500 ppm dose of Amistar top, followed 73.81% by 250 ppm while the lowest (72.14%) by 100 ppm of Amistar top (Fig. 4). Fungicide Tilt 250 EC (active ingredient: propiconazole) exhibited better mycelial growth inhibition of the studied fungus than Amistar top. The highest vegetative growth inhibition (89.42%) was observed for the fungus due to a higher dose (500 pm) of Tilt 250 EC, followed by 85.09% inhibition due to 250 ppm concentration (Fig. 4). However, the effect of Ridomil on vegetative growth inhibition was not promising, in which the highest mycelial growth inhibition (46.15%) of *F. fujikuroi* was measured at 750 ppm concentration, followed by 37.98% by 500 ppm and lowest 31.97% by 250 ppm concentration of the fungicide (Fig. 4). Our results are in agreement with the findings of Mallik et al. (2021) who tested the three concentrations (250 ppm, 500 ppm, and 750 ppm) of Ridomil gold MZ 68 wp, Jazz 80 wp, Tilt 250 EC and Amistar Top 325SC, in which Tilt 250 EC (500 ppm) showed the maximum mycelium inhibition (60.63%) of *Fusarium solani* under *in vitro* conditions. In another lab bioassay, ten fungicides were evaluated against several pathogenic fungi including *Fusarium moniliforme*, where Tall 250 EC (active ingredient: propiconazole) was found as an effective fungicide to inhibit the mycelial growth of the fungus (Chowdhury et al. 2015). Besides, Tilt 250 EC (500 ppm) was also found as an effective fungicide against *Fusarium oxysporum* under *in vitro* conditions (Ahmmed et al. 2021). Therefore, lab bioassay suggests that Tilt 250 EC (500 ppm) could be used as a potential fungicide to manage strawberry leaf blight disease causing by *Fusarium fujikuroi* species complex. However, further pot and field trial is necessary to confirm the effectiveness of Tilt 250 EC against the fungus.

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