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Characterization and Pathogenicity of *Fusarium* Species Associated with Wilt Disease of Cucumber

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Abstract

Cucumber is one of the highly demanded vegetable crops in Malaysia. However, several fungal diseases have been identified to attack the cucumber, causing a considerable loss in fruit yield. *Fusarium* wilt has been reported as one of the main causes of cucumber yield reduction. The study impinges to verify the virulence of all *Fusarium* isolates by performing pathogenicity test on cucumber to fulfill Koch's Postulates. Seventeen isolates of *Fusarium* species were recovered from infected cucumber fruits and leaves. All the isolates were identified and classified based on translation elongation factor (*tef1α*) and beta-tubulin (*β-tub*) sequence analyses. All *Fusarium* isolates were tested for pathogenicity test by soaking the root of 7 days-old cucumber seedling into 2 x 10^6 spore/ml of conidial suspension for 30 minutes. Sterile distilled water was applied as a control. The most virulent was recorded by *F. oxysporum* isolate D2505C with disease severity of 50%. Three isolates were identified as non-virulent with no visible wilt symptoms, which are *F. longipes* isolate D2504C and *F. incarnatum* isolates N2210C and N2212C. These results provide useful information on the diversity and pathogenicity of *Fusarium* species associated with wilt disease of cucumber in Malaysia.

Keywords: *Fusarium Wilt; Cucumber; Sequence Analysis; Virulence; Pathogenicity Test*

Introduction

The world agricultural development is increasing with time and technology. Along with the increment, the crops are still affected by various plant diseases including those caused by fungi. Cucumber is one of the economically important crops produced worldwide and is also affected by the fungal diseases. Commonly known as a primary model system of sex determination studies, cucumber has been well recognized in studying vascular system of the Cucurbitaceous family. Cucumber is generally domesticated from Indo-Gangetic plain. China is the world-leading producer of cucumber with 54.3 million tons production recorded in 2009 [1].

In Malaysia, cucumber is highly produced among the cucurbit crops. However, in 2016, the production of cucumber was reduced at 97.6 thousand tons compared to the previous year with 100.8 thousand tons. This resulted in 3.18% reduction of the production value [2]. The dwindling report was due to various diseases invading the crop. On the top of the diseases listed, *Fusarium* wilt was reported to contribute in the production loss. Several studies discovered that the species belonged to the genus *Fusarium* as saprophytes mycoflora, while others reported them as pathogenic [3]. The genus *Fusarium* is one of the major producers of secondary metabolite known as mycotoxin [4]. Currently, the knowledge on determining the virulence of different species in the genus is still vague. Nevertheless, the pathogenesis mechanisms of *Fusarium* wilt infection on cucumber plants are not well explained.

This study aims to identify the sequential symptoms caused by several *Fusarium* species isolated from wilt disease on cucumber. Despite the biological and agricultural importance of *Fusarium* species, the information on their diversity and pathogenicity is still inadequate. The main objectives of this study are to identify the molecular identification of *Fusarium* species isolated from wilt disease of cucumber and to determine their pathogenicity.

Materials and Methods

Fungal isolation

The infected cucumber samples were obtained directly from the field. The samples were collected from various locations in Peninsula Malaysia including Bachok (Kelantan), Bukit Rambai (Melaka) and Rembau (Negeri Sembilan). The tissue of leaves and fruits of the cucumber were cut into smaller size of about 1 cm x 1 cm. All tissue pieces were then sterilized by soaking into 10% Chlorox once and rinsed with sterile distilled water twice. All tissues were air dried on sterile filter paper before placed onto semi-selective medium, peptone pentachlonitrobenzene agar (PPA) and incubated for 5 days. The colonies were purified by streaking onto potato dextrose agar (PDA) and incubated for 5 days. Total isolates were stored in PDA slant at room temperature. For long term storage, the total isolates were cultured on pieces of sterile filter paper on PDA and incubated for 7 days. The cultures were then stored at -20^{0}C [5].

Figure 1: *Fusarium wilt infecting cucumber in Bachok, Kelantan. Infected plants demonstrate chlorotic and discoloration of the leaves (arrows).*

Microscopic morphology

The isolates were cultured onto Carnation Leaf Agar (CLA) and incubated for 7 days. Small drop of sterilized distilled water were put on the microscope slide. By using inoculum wire loop, small amount of fungal mycelia were scratched and placed in the water drop. Placed the cover slip on the slide and observed under compound light microscope. The *Fusarium* grown on water agar (WA) was also being observed by using the same method. The size, shape and number of septate of the macroconidia and microconidia were observed. The presence of chlamydospore, phialide and hypha were also being observed [4].

DNA extraction

All *Fusarium* isolates were cultured on PDA and incubated for 5 days. Small amount of mycelial sheet were scratched off the media by using pipette tip prior to DNA extraction. The DNA extraction was performed by using Ultra Clean® Microbial DNA isolation kit (MO-BIO, Carlsbed, CA, USA) according to the manufacturer procedures.

Amplification of *tef1α and β-tubulin* genes

The amplification of the fragments was performed using thermal cycler (Biometra®TProfessional). The total volume of 20 µl PCR master mix was prepared containing 4 µl of 5x 1.5 mM Green GoTaq buffer, 2 µl of 0.2 mM of deoxynucleotide triphosphate (dNTPs), 2 µl of 0.2 mM of magnesium chloride ($MgCl_2$), 0.1 µl of Taq Polymerase, 1 µl of DNA template, 8.9 µl nuclease free water. Two primer sets EF1 (5'-ATGGGTAAGGAGGACAAGAC-3') and EF2 (5'- GGAAGTACCAGTGATCATGTT-3') [6] and Beta-tubulin primer sets T1 (5'-AACATGCGTGAGATTGTAAGT-3) and T2 (5'-TAGTGACCCTTGGCCCAGTTG-3') [7] were used to amplify *tef1α* and *β-tubulin*. The PCR program of *tef1α* gene was held through the following cycles; initial denaturation at 94^0C for 90s, 35 cycles of denaturation at 95^0C for 35s, annealing at 57^0C for 55s, extension at 72^0C for 90s and final extension at 72^0C for 10 minutes [8]. For *β-tubulin*, the PCR program was held through the following cycles; initial denaturation at 94^0C for 1 minute, 35 cycles of denaturation at 94^0C for 35s, annealing at 58^0C for 2 minutes, extension at 72^0C for 1 minute, final extension at 72^0C for minute and soak at 4^0C [9].

Gel electrophoresis

To quantify the PCR product, gel electrophoresis was conducted by using 1.2% final concentration of agarose gel. The gel was stained with Fluorosafe dye (1st Base Company, First Base Laboratories Sdn. Bhd. Seri Kembangan, Selangor, Malaysia). A 100bp ladder was used as the marker of the fragments size. The gel was the captured with G:BOX Syngene under ultra violet (UV) radiation.

Nucleotide sequence and phylogenetic analysis

The PCR products were purified and sequenced by MyTACG Bioscience Company. The purified PCR products were sequenced by using ABI3730XL sequencer. The sequences were analyzed and compared to the database from National Center of Biotechnology Information (NCBI) at https://www.ncbi.nlm.nih.gov for Basic Local Alignment Search Tool (BLAST). The sequences were aligned for phylogenetic analysis using Clustal W in Molecular Evolutionary Genetics Analysis version 7.0 (MEGA 7.0) [10]. The Maximum Likelihood analysis by using Tamura-Nei model generated a phylogenetic relationship from aligned sequences for each data set. About 1000 replications of bootstrap analyses supported the phylogenetic groupings.

Conidial suspension

Fusarium isolates were grown on PDA for five days at room temperature 30 ± 1^0C. Ten ml of sterilized distilled water was poured on the PDA plate containing fungal culture. By using micropipette tip, the fungal mycelia were scratched to detach the mycelia sheet and conidia off the media. The 10 ml inoculum was then transferred into 500 ml conical flask. Dilution was done by adding sterile distilled water (dH_2O) into the 10 ml of inoculum forming a 200 ml final concentration of 2 x 10^6 conidia/ml by the aid of haemacytometer [3]. Any appearance of mycelial sheets was filtered.

Plants preparation

The cucumber seeds were surfaced sterilized by soaking them in 10% of Chlorox and washed twice with sterile distilled water (dH_2O). The seeds were then air-dried on sterile filter paper prior sown [11]. The soils were prepared by mixing of top soil, river sand and manure. A total of 1 kg soil with the mixture ratio of 3:2:1 (top soil: manure: river sand) was autoclaved at 121^0C for 15 minutes and cooled down overnight [12]. The soils were poured into 8 cm x 10 cm polybag before seed sown.

Root inoculation

Roots of 7 days old plants were washed with tap water. The roots were air dried and wounded by cutting 1 cm long [13]. After the roots are dried, they were carefully soaked in 500 ml flask containing fungal inoculum for 30 min on the orbital shaker at 100 rpm. The inoculated roots were then transplanted into poly bags [14]. The cucumber roots treated with sterile distilled water served as control. All treatments were performed in randomized complete block design (RCBD) inside plant house of Biology Department of Science Faculty, UPM. Three replications were prepared for each isolate. The plants were grown at 12/12 hr of 32 ± 1^0C days and 28 ± 1^0C nights with humidity of 72% for 21 days observation. The disease severity of all isolates was analyzed using analysis of variance (ANOVA) in the Statistical Package for Social Sciences (SPSS) software version 24.0. The comparison among isolates were made by using Duncan's multiple test ($p < 0.05$).

Disease assessment

After 21 days of inoculation, the emergence of any particular *Fusarium* wilt symptoms was assessed using modified disease scale of Schoonhoven and Pastor-Corrales, Raupach., *et al*. [15,16] as stated in table 1.

Scores	Inference[a]
0	Seed germinated no symptom of wilt.
1	Seed germinated, 1 - 24% of leaves showing slight chlorosis.
2	Seed germinated, abnormal growth with 25 - 49% of leaves showing chlorosis and/or curvature.
3	Seed germinated, abnormal growth with 50 - 74% of leaves wilting, chlorosis and/or limited necrosis.
4	Seed not germinated or seed germinated, abnormal growth with ≥ 75% of the leaves showing wilt symptoms.

Table 1: *Disease severity scales for Fusarium wilt assessment*

[a]*wilt symptoms-wilting, severe stunting and necrosis with premature defoliation often resulting in plants dying.*

The disease severity index was calculated for each isolate according to the parameters in the disease scale [17];

$$DSI = \frac{\Sigma (A \times n)}{\Sigma (B)} \times 100$$

A: disease scales

n: number of plants in specific scale

B: total number of plants

Results and Discussion

Phylogenetic analysis

tef1α and *β-tub* genes revealed a highly informative and frequently known as phylogenetically inferred between species and conveys a functional diversification in different evolutionary mechanisms in most of fungal lineages [18]. A total of 17 isolates recovered from infected cucumber were amplified by the combination of both genes and identified them as *F. solani* (eight isolates), *F. incarnatum* (six isolates), *F. oxysporum* (one isolate), *F. proliferatum* (one isolate) and *F. longipes* (one isolate) (Figure 2). The sequences of total isolates were subjected to *Fusarium* database in the NCBI and comparison by using BLAST revealed the closely related sequences as well as the percentage of homology ranged from 97 - 100% of similarity.

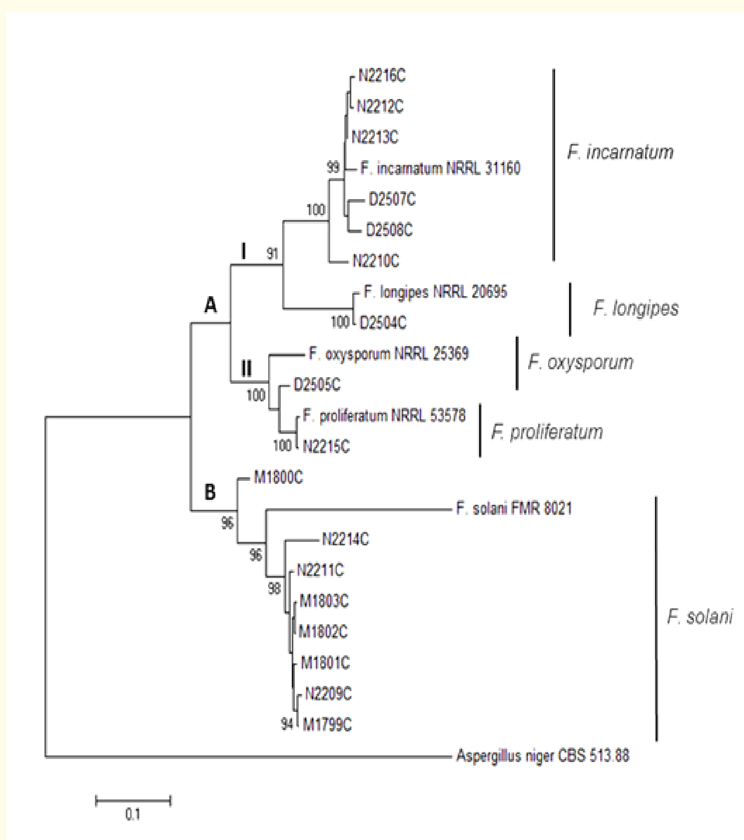

Figure 2: *Phylogenetic tree generated by Maximum Likelihood (ML) analysis of 17 Fusarium isolates of tef1α and β-tubulin gene sequence by using Jones-Taylor-Thornton model.*

Based on the phylogenetic tree, two main clades were generated- clade A and clade B. Clade A is split up forming two sub-clades; I and II. Sub-clade I consists of all isolates belonging to *F. incarnatum* and a single isolate identified as *F. longipes*. Six isolates belonged to sub-clade I was identified as *F. incarnatum*. This dominantly non-virulent fungus was classified as section Arthrosporiella. Sub-clade I consists of *F. longipes* as single isolate belonging to the species. Phenotypically, *F. longipes* was identified of having distinct morphological characteristic than any other *Fusarium* isolates of the study. This includes the pigmentation and shape of the macroconidia. *F. longipes* was classified as Type A trichothecene species, commonly known mycotoxin produced by the genus *Fusarium*. Other than *F. longipes, F. poae* and *F. sporotrichioides* also belonged to the same type [6]. Sub-clade II presents all of isolates identified as *F. oxysporum* and *F. proliferatum*.

This result relatively complement to previously reported by Nur Baiti., *et al.* [19] as *F. oxysporum* and *F. proliferatum* belong to the section Elegans and Liseola of *Gibberella fujikuroi* species complex (GFSC). *Fusarium oxysporum* and *F. proliferatum* are from different sections but shared the same clade of phylogenesis. The *F. oxysporum* species complex was considered as monophyletic that presented genotypically heterogenous morphospecies [7]. Meanwhile, in clade B consists of all isolates identified as *F. solani.* In this clade, all *F. solani* isolates were having a high polymorphism among each other by 95% and thus showing this partial belong to section Martiella. Generally, the distribution of all isolates according to the phylogenetic analysis revealed the evolutionary relationship among the 17 isolates decent according to the species. The nodes of the phylogeny analysis depict the similarities and differences based on genetic and physical characteristics. From the analysis, we could classify that isolates belong to clade B fall into virulent species. However, the isolates in clade A share most similarities on the morphological characteristic but they are all subsequently descending according to be different based on virulence. Sub-clade I consists of all non-virulent and sub-clade II is virulent.

Pathogenicity test

The highest virulent was produced by *F. oxysporum* isolate D2505C with 50% disease severity recorded (Figure 3). There are several historical studies on *F. oxysporum* as the causal agent of *Fusarium* wilt disease often invading the cucurbits [3]. Relatively, this phenomenon was almost similar by *F. oxysporum* in this study, which resulted in a slightly low strength of pathogenicity with 50% disease severity. Three isolates were identified as non-virulent with no visible wilt symptoms, which were *F. longipes* isolate D2504C and F. incarnatum isolates N2210C and N2212C (Figure 3). *Fusarium incarnatum* formerly known as *F. semitectum* [23], was classified as to may exist as saprophyte or opportunistic pathogen. It distributes well in the soil or even on the host biding around to be pre-disposed in condition such as drought. Thus, the result of present study was precisely consistent with a pathogenicity test of *F. incarnatum* on cucumber plants conducted in Kermanshah province, Iran. The findings resulted as *F. incarnatum* as a non-virulent due to presence less of lesion on the stem indicating the wilt disease [3].

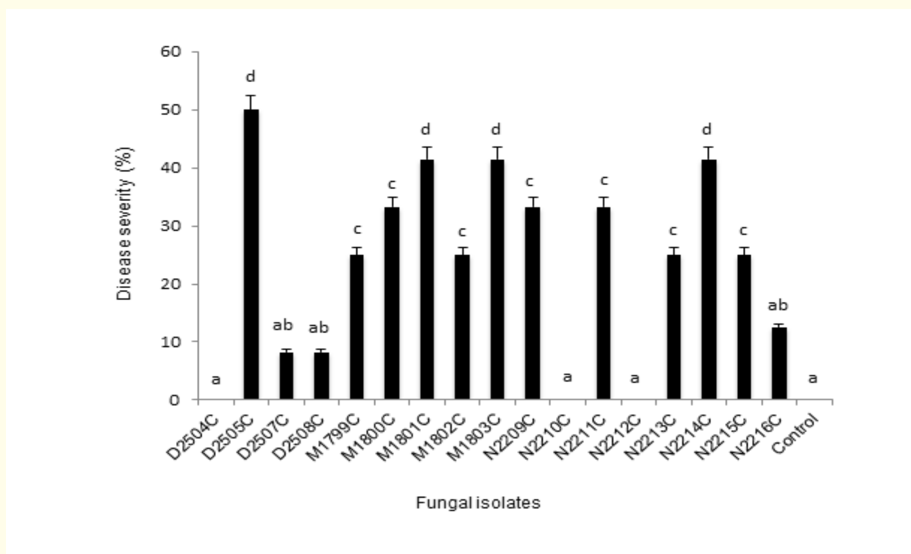

Figure 3: *Disease severity of Fusarium isolates on cucumber.*

The symptoms were less obvious at the shoot structures compared to the roots. Phytopathologist indicated that this is due to the rapid root extension of the plant that lowers the probability of infection [20]. This mechanism reduced the frequency of the root encountering the pathogen as the soil may be offset to some degree of extension. To support strongly this, Ondrej*., et al.* [21] previously reviewed that different isolates result in a significantly distinct virulence in respect to the methods of inoculation.

The symptoms were observed as early as 9 days post inoculation (dpi) by the appearance of chlorotic and discoloration of the leaves. Some of the plants displayed wrinkled leaves prior to the formation of chlorosis. At 11 dpi, the plants inoculated with *F. solani* isolate M1801C started to be stunted with no increase in leaves total area and stem length developments. According to the study conducted by Caroline and Martijn [22], these are the initial symptoms of vascular wilt disease that subsequently followed by stunting, yellowing of the lower leaves and progressive wilting defoliation followed by death of the plant.

Upon colonization, *Fusarium* sp. will cause the vascular tissue to turn brown or necrosis in the cross-section of the stem. The observation on vascular tissues upon colonization of *Fusarium* species was proportional to the disease severity. *F. solani* and *F. oxysporum* showed significant ($p < 0.05$) effect on the vascular tissues compared to the control where the tissue cells were shrunken and necrotic lesions were observed. The massiveness of the root branches was also reduced (Figure 4).

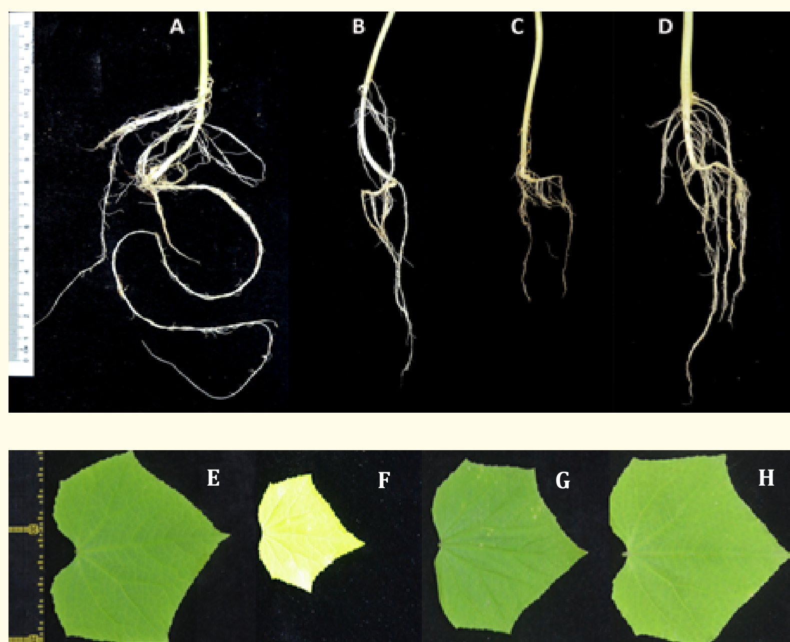

Figure 4: *Cucumber roots and first true leaf after 21 days inoculation. A and E: control, B and F: inoculated with F. solani, C and G: inoculated with F. oxysporum, D and H: inoculated with F. proliferatum.*

Three main features what make up *Fusarium* are the macroconidia, microconidia and chlamydospore. Commonly known as the asexual spores, these conidia are responsible in the process of fungal dissemination and pathogenesis. The macroconidia has a crescent and sickled shape with curvature at the center and septates of about three to four. The microconidia usually appear in ellipsoidal and oval shape. Smaller in size when compare with macroconidia. The chlamydospore is the resting and survival spores. Thick cell wall surrounds the chlamydospore and it usually could be observed as single, double and chained cells. Hypha structures made up the most appearance of nearly all filamentous fungi. This functional structure however is poorly understood. But generally, for *Fusarium* sp., the growth and extension of structural hypha plays important role in constituting multicellular colonies. The development from hypha to mycelia occurs in two processes; (i) tip extension and branching from an individual hypha which entail the growth axis and (ii) fusion of hypha to interlink the hyphal networks [24]. Even though, it was unveiled that the role of hypha structure had not essentially crucial during pathogenesis, but

literally it involves during invasive growth and penetration through the root tips. Evidently, in present study we could capture varieties of structural hypha from all orthologous species. *F. oxysporum, F. proliferatum* and *F. longipes* indicated short polyphialides, whilst *F. solani* and *F. incarnatum* were both long monophialides (Figure 5). The short polyphialides are where they extended and forming the conidiophore that hold and attach the conidia. At this area where we could see the accumulation of macroconidia and microconidia bunches. All species were observably clear septation along the hyphal structures regularly. These results are concordant with the microscopic characteristics of *Fusarium* sp. isolated from fruit rot disease of tomato in Selangor, Malaysia [25].

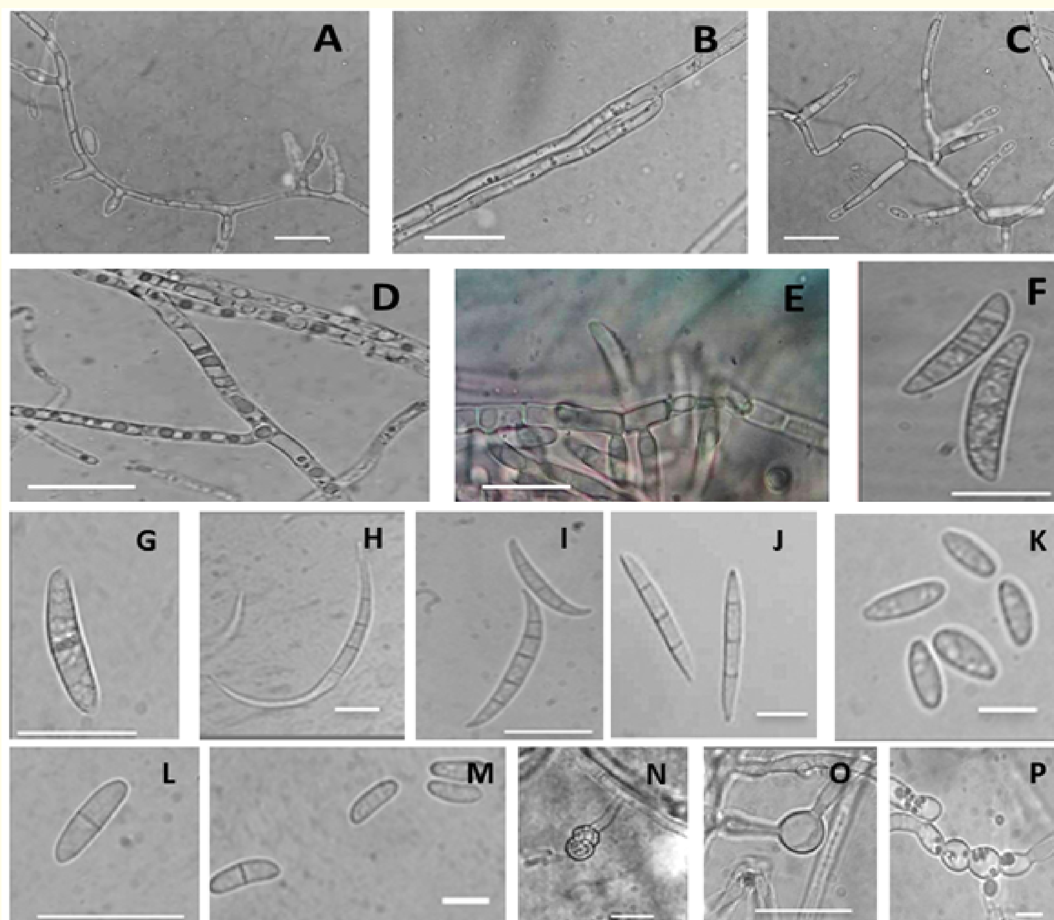

Figure 5: *Micromorphological characteristics of Fusarium species. Hypha of A: F. oxysporum, B: F. solani, C: F. proliferatum, D: F. incarnatum and E: F. longipes. Macroconidia of F: F. oxysporum, G: F. solani, H: F. longipes, I: F. incarnatum and J:F. proliferatum. Microconidia of K: F. oxysporum, L: F. incarnatum and M: F. solani. Chlamydospore of N: F. solani, O: F. oxysporum and P: F. incarnatum. (Scale bar: 10 µm).*

Conclusion

As a conclusion, *F. oxysporum* and *F. solani* were identified as the main causal pathogens contributing to wilt disease in cucumber based on the pathogenicity test. The isolates disease severity varies toward the plants with multiple and sequential wilt symptoms. The inquiry of this study leads to the useful information on the diversity and pathogenicity of *Fusarium* species associated with wilt disease of cucumber in Malaysia. With all these information, the regulation of functional genes induced upon inoculation of pathogenic *Fusarium* species on cucumber would be beneficial to acquire the pathogenesis descriptions in the future.

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Conflict of Interest

No any financial and conflict of interest exists.

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