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PATHOGENICITY OF *COLLETOTRICHUM* SPECIES CAUSING ANTHRACNOSE DISEASE ON RED CHILLI IN TERENGGANU, MALAYSIA

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ABSTRACT

Colletotrichum genus can survive in many plant parts and remain dormant for a long time. This situation triggered a problem in the field, storage and marketing sections since it becomes a source of inoculum to spread anthracnose disease. Therefore, information on fungal pathogens causing anthracnose disease on chilli and their pathogenicity level is important for breeding purposes especially for strategies management control of the disease. This study was aimed to identify fungal pathogens and its pathogenicity causing anthracnose symptoms on red chilli isolated from seven districts in Terengganu, Malaysia. For this purpose, fungi associated with anthracnose disease were isolated and identified using morphology and molecular characteristics. Then, the pathogenicity test was evaluated for all the isolates to determine its pathogenicity. A total of 19 fungal isolates were morphologically identified as *Colletotrichum* species. From these, eight isolates have been identified as *Colletotrichum capsici* isolated from district Kuala Nerus and Marang; and 11 isolates have been identified as *C. acutatum* isolated from districts Kuala Nerus, Kuala Terengganu, Kemaman, Dungun, Besut and Setiu. All the isolates were able to cause infection to the chilli pod with different levels of disease severity ranging from 53.3% to 100%. Different species of *Colletotrichum* genus was able to infect many different parts of chilli plant and other hosts. The data of this study can provide information on correct identification of the pathogens, predict the occurrence of anthracnose disease, and help in management of the disease.

Keywords: Plant disease, chilli, *Colletotrichum*, morphology, molecular, pathogenicity.

INTRODUCTION

Red chilli or scientifically known as *Capsicum annuum* lead the major production compared to other varieties (Sahitya *et al*., 2014). The crop has been planted on large scale worldwide for food industry (Hussain and Abid, 2011), pharmaceutical industries and cosmetic industries. According to Sahitya *et al*. (2014), many beneficial effects can be obtained by consuming chilli such as enhancing blood circulation, reducing platelet aggregation, reduce calories and cancer risk, and lessening pain by releasing endorphins in the body.

Good quality chilli is important to ensure it can be sold at high price and gain more profit. However, occurrence of pest and diseases problem at pre-harvest stage of chilli production is quite challenging which leads to heavy losses (Hussain and Abid, 2011). Chilli is susceptible to many diseases' infections caused by fungi, virus and bacteria, and become a major constraint to many producers. In developing countries, poor transportation practices and storage facilities can increase the damage of the yield (Saxena *et al*., 2016). Previous studies reported that anthracnose disease caused by *Colletotrichum* species is considered as main fungal disease problem at pre-harvest stage of chilli crop. During infection, different *Colletotrichum* species can be involved in the same host plant (Freeman *et al*., 1998; Cannon *et al*., 2000). Three species namely *C. acutatum, C. capsici, C. gloeosporioides* are commonly reported as anthracnose pathogens on chilli in Indonesia, Thailand, Taiwan and Vietnam (Voorrips *et al*., 2004; Than *et al*., 2008; Manandhar *et al*., 1995 and Don *et al*., 2007). In addition, different *Colletotrichum* species can infect different parts of the

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chilli plant (Kim *et al.*, 2004). *Colletotrichum capsici* was frequently infected the red chilli fruits while *C. acutatum* and *C. gloeosporioides* were more widespread on immature chilli fruits (Harp *et al*., 2008). Occurrence of anthracnose disease cause destructive loss at pre-harvest and post-harvest stage (Bosland and Votava, 2003; Sahitya *et al*. 2014). Loss of yield can reduce the farmer's profit especially in developing countries such as Pakistan, India, Thailand, Mexico including Malaysia (Freeman *et al*., 1998; Than *et al*., 2008; Shahbazi *et al*., 2014).

Genus *Colletotrichum* is regarded as an important plant pathogen and many crops have been reported to infect this species. The pathogen can survive in the seed or on the surface of the seed in the form of acervuli and micro-sclerotia (Pernezny *et al*., 2003). Heavy colonization of the *Colletotrichum* species in the seed can disintegrate the parenchymatous layers of the seed coat which eventually deplete the food material in endosperm and embryo. The appressoria produced on immature fruits can persist dormant until the fruit is harvested and stored. This situation can cause a huge problem in storage and marketing sections since small infection of the pathogens will reduce the quality of chilli and become a source of inoculum to spread the disease. Therefore, this study was conducted to identify the fungal pathogens associated with anthracnose symptoms on red chilli isolated from seven districts in Terengganu, Malaysia. Since anthracnose is considered a destructive disease in chilli plant, identification of fungal pathogens is important for breeding purposes especially to strategies management of anthracnose disease.

MATERIALS AND METHODS

Fungal Isolation: Infected chilli pods with anthracnose symptoms were collected from seven districts in Terengganu, Malaysia was cut into small pieces, $5mm^3$. The infected tissues were dipped into 1% sodium hypochlorite and rinsed three times with sterile distilled water. After that, the tissues were air-dried onto the sterile filter paper before being placed on potato dextrose agar (PDA) plate. All the plates were incubated at room temperature (28 ± 2°C) for three days until appearance of fungal mycelium. Then, the mycelia undergo a single colony process to obtain a pure culture.

Morphological and Molecular Characterization: The obtained pure culture was regarded as one fungal isolate and labeled with a code number. The isolate was subculture onto PDA media to observe morphological characteristics such as colony colour and pigmentation. Then, the mycelia of the isolates were morphologically identified using digital stereo binocular microscope to observe the spores. All the fungal isolates were identified based on characteristics described by Than *et al*. (2008).

For molecular characterization, the fungal ITS gene was amplified using universal primers ITS1 and ITS4 (White *et al*., 1990). A total reaction volume was 25µl containing DNA template, primer, deoxynucleotides triphosphates (dNTP), DNA polymerase, buffer and water (double distilled H_2O) following the manufacturer's protocol. The PCR was performed as follow; 1 cycle of initial denaturation for 120 seconds at 98°C, 25 cycles of denaturation for 15 second at 98°C, annealing for 30 seconds at 60°C, extension for 30 seconds at 72°C and final extension for 600 seconds at 72°C. The PCR products were purified using the standard method before sequence using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The obtained nucleotide sequences were searched through the BLAST program for homologous fungal sequences at http://www.ncbi.nlm.nih.gov.

Pathogenicity Test: All fungal isolates obtained during isolation process were used in pathogenicity test. For this purpose, healthy chilli surface was sterilized with 3% sodium hypochlorite and rinsed three times with distilled water. All the chilli pods were blotted dried using a sterile tissue paper before being inoculated with fungal isolates. The chilli pods were wounded at 1 mm depth with a sterilized scalpel before placed a PDA plug containing fungal isolate facing to the wound surface. For controls, the healthy chilli pods were inoculated with PDA plugs without fungal isolate. All the chilli pods were arranged into a container and incubated at room temperature. Appearance of symptoms were evaluated at 3 days interval for 12 days after inoculation based on the disease severity scale adopted from Shahbazi *et al*. (2014) with some modification. The scale was as follow, 0=the fruit was healthy, 1= 10% of fruit area was infected, 2= 25% of fruit area was infected, 3=50% of fruit area was infected, 4= 75% of fruit area was infected and 5=100% of the fruit was infected. Then, percentage of disease severity was calculated as follow:

$$\text{Disease index} = \frac{\sum (\text{ds x n})}{(\text{N x Z})} \times \mathbf{100}$$

Where,

ds= disease severity

n= the number of infected plants with disease

N= total number of plants

Z= maximum disease severity

Experimental Design and Statistical Analysis: The experimental design used in this study was completely randomized design (CRD) with three replicates. The data were analysed using analysis of variance (ANOVA), using Tukey's test for pairwise comparison of mean values (p=0.05). All the tests were computed by using Statistical Package for the Social Sciences (SPSS) version 20.

RESULTS AND DISCUSSION

Isolation and Identification of Fungal isolates: Many farmers in Terengganu faced challenging problems during chilli cultivation especially in monsoon season. Occurrence of anthracnose disease can cause extensive losses of chilli during pre- and post-harvest stage, either in the fields or during storage every year. During sampling, the anthracnose symptoms are found more on chilli pod compared to chilli leaves which showed sunken lesions and containing spores of fungal species. Mahasuk *et al*. (2009) reported that the anthracnose symptoms can be found on both plant and leaves, on legumes and other crops. In this study, a total of 19 fungal isolates were morphologically identified as *Colletotrichum* species have been isolated from chilli pod with anthracnose symptoms (Figure 1A and Figure 1B). All the isolates were collected from seven districts namely Kuala Nerus (eight isolates), Kuala Terengganu (three isolates), Kemaman (one isolate), Dungun (two isolates), Marang (one isolate), Besut (two isolates) and Setiu (two isolates) in the state of Terengganu, Malaysia (Table 1). Based on morphological characteristics, all the fungal isolates show white to grey colour with dark green at the centre of cottony mycelium (Figure 1C and Figure 1D). It also produced diurnal zonation of dense and sparse development of aerial mycelium (Figure 1E and Figure 1F). Under 40X magnification, the mycelium was dense and filamentous. Some of the isolates produce dark brown and rounded acervuli. In all the isolates, it has abundance of falcate shape of conidia which were slightly tapering towards the end (Figure 1G-Figure 1H). For some of the isolates, the setae were also observed (Figure 1I). These microscopic characteristics of *Colletotrichum* species were similar as reported by Than *et al*. (2008) and Prajapati *et al*. (2020).

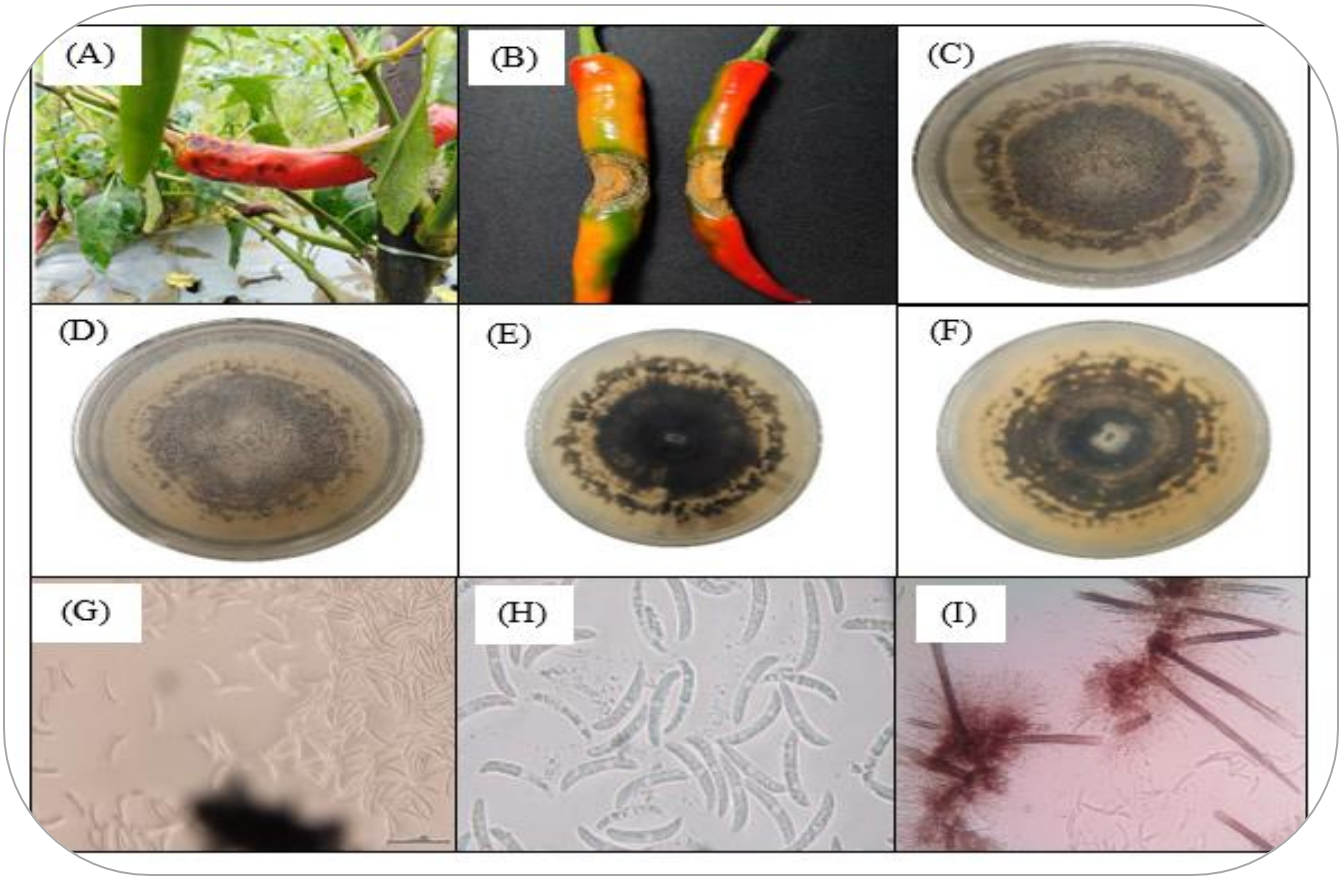

Figure 1. Antracnose symptoms on chilli pod during sampling (A) and (B), top colony colour of fungal isolates in (C) and (D), diurnal zonation of aerial mycelium from bottom colony in (E) and (F), falcate shape conidia in (G) and (H) and setae with needle like in (I).

Table 1. Fungal identification using morphology and molecular characteristics.

Code of isolates	Morphologically identified species	Molecular identified species (Percentage of sequence similarity in GenBank)	District
FP1	*Colletotrichum* sp.	*Colletotrichum capsici* (100%)	Kuala Nerus
FP2	*Colletotrichum* sp.	*Colletotrichum capsici* (100%)	Kuala Nerus
FP3	*Colletotrichum* sp.	*Colletotrichum capsici* (100%)	Kuala Nerus
FP4	*Colletotrichum* sp.	*Colletotrichum capsici* (100%)	Kuala Nerus
UMT01A	*Colletotrichum* sp.	*Colletotrichum capsici* (100%)	Kuala Nerus
UMT02A	*Colletotrichum* sp.	*Colletotrichum capsici* (100%)	Kuala Nerus
UMT03A	*Colletotrichum* sp.	*Colletotrichum acutatum* (100%)	Kuala Terengganu
UMT04A	*Colletotrichum* sp.	*Colletotrichum acutatum* (100%)	Kuala Terengganu
UMT06A	*Colletotrichum* sp.	*Colletotrichum acutatum* (100%)	Kemaman
UMT07A	*Colletotrichum* sp.	*Colletotrichum acutatum* (100%)	Dungun
UMT08A	*Colletotrichum* sp.	*Colletotrichum acutatum* (100%)	Dungun
UMT10A	*Colletotrichum* sp.	*Colletotrichum capsici* (100%)	Marang
UMT11A	*Colletotrichum* sp.	*Colletotrichum acutatum* (100%)	Besut
UMT12A	*Colletotrichum* sp.	*Colletotrichum acutatum* (100%)	Besut
UMT13A	*Colletotrichum* sp.	*Colletotrichum acutatum* (100%)	Kuala Nerus
UMT15A	*Colletotrichum* sp.	*Colletotrichum acutatum* (100%)	Setiu
UMT16A	*Colletotrichum* sp.	*Colletotrichum acutatum* (100%)	Setiu
UMT17A	*Colletotrichum* sp.	*Colletotrichum acutatum* (100%)	Kuala Terengganu
UMT19A	*Colletotrichum* sp.	*Colletotrichum capsici* (100%)	Kuala Nerus

Major infection of anthracnose disease on chilli is caused by *Colletotrichum* species. It has broad range of hosts such as legumes, cereals, perennial crops, vegetables and tree fruits (Bailey and Jeger, 1992). The production of anthracnose symptoms on chilli plant will trigger an alarm signal to farmers because the disease can spread very quickly. In addition, this genus is considered as most important pathogens that cause latent infection (Jeffries *et al*., 1990). In many cases, most of the infections will remain latent and develop the symptoms on the ripen fruits whereas physiological changes already occurred in the host (Bailey and Jeger, 1992).

Through molecular study using ITS region, eight isolates have been identified as *Colletotrichum capsici* with percentage identity, 100% isolated from district Kuala Nerus and Marang. A total of 11 isolates have been identified as *C. acutatum* with percentage identity, 100% isolated from district Kuala Nerus, Kuala Terengganu, Kemaman, Dungun, Besut and Setiu (Table 1). Different species of *Colletotrichum* can be found to be associated with anthracnose disease on chilli. According to the previous study, several *Colletotrichium* species such as *C. acutatum*, *C. capsici*, *C. coccodes* (Ranathunge *et al*., 2012; Saxena *et al*., 2014, Damm *et al*., 2012 and Liu *et al*., 2013) and *C. gloeosporioides* have been reported to cause anthracnose disease on chilli. However, this study only identified two fungal species associated with anthracnose symptoms on red chilli namely *C. capsici* and *C. acutatum*. According to Kim *et al*. (2004), different species of *Colletotrichum* is able to infect different parts of the chilli plant. For example, *C. gloeosporioides* and *C. acutatum* commonly infect chilli fruits at all developmental stages while *C. coccodes* and *C. dematium* commonly found on infected leaves or stems. In addition, *C. capsici* is prevalent in red chilli fruits while *C. acutatum* and *C. gloeosporioides* normally found in immature chilli fruits (Harp *et al*., 2008). According to Peres *et al.* (2004) and Whitelaw-Weckert *et al*. (2007), some *Colletotrichum* species has various respond against different control measures. Thus, identification of *Colletotrichum* species is important in disease management control and this information can be used in breeding purposes.

Pathogenicity Test: Table 2 shows average percentage of disease severity (DS) for all *Colletotrichum* isolates. In this study, *Colletotrichum* isolates were able to cause infection to the chilli pod with different level of disease severity. As early as day 3, the inoculated area on the chilli pod shows small, brown and necrotic spots. As day increased, the inoculated area become darker and larger

lesion. The sunken necrotic areas of inoculated place were covered with many black acervuli on the surface. Among the isolates, four isolates of *C. acutatum* and one isolate of *C. capsici* recorded 100% disease severity on the chilli pod. Other isolates recorded disease severity ranged between 53.3% to 95.56%. The lowest disease severity was recorded on *C. capsici* (FP1) isolated from district Kuala Nerus. After re-isolation, the similar symptom was obtained, thus Koch's postulate was fulfilled. At day 12, control chilli pod did not show any disease symptoms. All percentage of disease severity were significantly different with the control ($p<0.05$).

Table 2. Percentage of disease severity for *Colletotrichum* species isolated from anthracnose disease on chilli.

Fungal Code	*Colletotrichum* species	Disease Severity + SD
FP1	*C. capsici*	53.30 + 1.44 ^{b}
FP2	*C. capsici*	60.00 + 1.63 ^{bc}
FP3	*C. capsici*	57.80 + 1.48 ^{bc}
FP4	*C. capsici*	57.80 + 1.48 ^{bc}
UMT01A	*C. capsici*	62.22 + 3.85 ^{bcd}
UMT02A	*C. capsici*	57.78 + 3.85 ^{bc}
UMT03A	*C. acutatum*	95.55 + 3.85 ^{ef}
UMT04A	*C. acutatum*	95.55 + 3.85 ^{ef}
UMT06A	*C. acutatum*	80.00 + 6.67 ^{de}
UMT07A	*C. acutatum*	75.56 + 7.69 ^{cd}
UMT08A	*C. acutatum*	100.00 + 0.00 ^{f}
UMT10A	*C. capsici*	55.56 + 15.39 ^{b}
UMT11A	*C. acutatum*	95.56 + 3.85 ^{ef}
UMT12A	*C. acutatum*	100.00 + 0.00 ^{f}
UMT13A	*C. acutatum*	80.00 + 17.64 ^{de}
UMT15A	*C. acutatum*	95.56 + 7.69 ^{ef}
UMT16A	*C. acutatum*	100.00 + 0.00 ^{f}
UMT17A	*C. acutatum*	100.00 + 0.00 ^{f}
UMT19A	*C. capsici*	100.00 + 0.00 ^{f}
Control	-	0.00 + 0.00 ^{a}

Mean values with the same letter in the same column are not significantly different at $p<0.05$.

This study shows that different *Colletotrichum* isolates recorded different pathogenic level on the chilli pod. The variation of pathogenic level in the isolates might be due to genetic variability of the fungal pathogen. According to McDonald (1997), the genetic of plant pathogenic fungi will evolve due to mating systems, gene flow, mutation or migration, population size and selection. In addition, *Colletotrichum* species complex comprise of individual species that may infect different host plants, and some may produce apparent disease symptoms at different times (De Silva *et al*., 2017). A study conducted by Noor and Zakaria (2018) reported that different species of *Collectotrichum* namely *C. truncatum*, *C. scovillei*, *C. fioriniae*, *C. fructicola*, and *C. siamense* exhibits different pathogenic level on red chilli in Malaysia by using the same method. Most of the symptoms appeared on the chilli pod were sunken circular or angular lesions caused by *Collectotrichum* spp. Due to this infection, multiple lesions will be produced on the chilli pod thus causing the fruit rot (Saxena *et al*., 2016). The aggressiveness of fungal pathogen to infect the chilli plant is relate to the compatibility of plant-pathogen interactions that is often governed by the gene-for-gene model in many pathosystems (Flor, 1971). Than *et al*. (2008) suggested that this situation happened due to continuous co-evolutionary change in both plant and pathogen. According to Taylor and Ford (2007), some pathogen populations are known to be pathogenetically diverse which was suggested due to continuous generation of novel pathogenic variations. Saxena *et al.* (2016) reported that anthracnose on chilli can be spread through seed whereas the fungus can grow in or on seed in the form of acervuli and microsclerotia. Therefore, if the disease management is not done properly especially in the early stages of the chilli plant, the infected part of the plant will be the main inoculum to spread the anthracnose disease in the field. In addition, imported chilli seeds need to be carefully controlled to avoid the spread of this disease with new species that affect the control strategies in the future.

CONCLUSION

Production of chilli in Malaysia is facing a problem due to fungal diseases thus reducing the crop yield. Anthracnose disease caused by *Colletotrichum* species are mostly

reported as the most devastating and harmful fungal diseases during planting and post-harvest stages of chilli cultivation. The pathogenic isolates of *Colletotrichum* species can spread faster and infect healthy chilli plant as well as the other crops. The potential of this species to cross infection is possible since it has a broad host range. Therefore, information on correct identification of *Colletotrichum* species and its pathogenic level is important for pathological, plant quarantine and breeding purpose. Correct identification of causal pathogen can help in management control of anthracnose disease in chilli plantation.

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