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Comparative Morphology and Characterization of *Colletotrichum* Isolates Occurring on Cocoa in Malaysia

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ABSTRAK

Pemencilan daripada daun-daun koko yang menunjukkan gejala "shot-hole", hawar dan bintik daun, juga daripada putik dan buah yang reput menghasilkan Colletotrichum gloeosporioides. Tiada perbezaan jelas dari segi ciri-ciri kultur dan morfologi dicatatkan daripada pemencilan-pemencilan yang berbeza. Kulat didapati hidup dan membentuk spora dengan baik pada 30°C. Agar Ekstrak Daun Koko (CLEA) merupakan medium yang sesuai untuk pertumbuhan miselium manakala Agar Kentang Dekstros (PDA) menggalakkan pensporulaan. Daun-daun koko dan juga buah yang dicederakan mudah dijangkiti oleh C. gloeosporioides. Anak benih koko berumur tiga minggu dan putik menunjukkan peringkat yang paling rentan terhadap jangkitan.

ABSTRACT

Isolation from cocoa leaves showing symptoms of shot-hole, blight or irregular leaf spot and from cherelles and pod rot yielded Colletotrichum gloeosporioides. No distinct differences in cultural and morphological characteristics were noted between the various isolates. The fungus was found to grow and sporulate well at 30°C. Cocoa Leaf Extract Agar (CLEA) was the best medium for mycelial growth while Potato Dextrose Agar appeared to favour sporulation. Both cocoa leaves and injured pods were liable to infection by C. gloeosporioides. Three week old cocoa seedlings and cherelles were noted as the most susceptible stages.

Keywords: ***Colletotrichum gloeosporioides*, cocoa, temperature, culture media, infectivity**

INTRODUCTION

Leaf spot and pod rot of cocoa (*Theobromae cocoa*, L) incited by *Colletotrichum gloeosporioides* which were first reported to occur in Malaysia in 1975 by Lin and Liew (1975), cause severe blighting and rotting of cherelles and immature pods. However, leaf spot disease and pod rot caused by *C. gloeosporioides* have yet to become a major disease problem in Malaysia even though they have been recorded to be serious in several cocoa growing countries in the world (Thorold, 1975; Dakwa and Danquah, 1978). Although several workers have studied the taxonomy, morphology and biology of the pathogen (Stoneman, 1898, Shear and Wood, 1913; Burger, 1921; Simmonds, 1965; McDonald, 1926; Wastie and Shanker, 1970; Arx, 1970), very little information on the etiology of this disease under local conditions is available. Since this information is required for the formulation of a comprehensive disease control programme for *Colletotrichum* leaf spot and pod rot of cocoa in this country, this study focuses on the occurrence of the disease in Malaysia; and to establish the identity of the pathogen, its infectivity on different parts of the plant and its symptoms.

MATERIALS AND METHODS

Isolation of the pathogen

Cocoa leaves showing symptoms of leaf spot disease were collected from both cocoa seedlings and mature cocoa trees grown in the Cocoa Unit of Universiti Pertanian Malaysia. In addition to infected cocoa leaves, cherelles, immature pods as well as mature pods suspected of being infected by *Colletotrichum* were also collected. Pieces of the infected leaf, 3mm × 3mm in area, and pod tissues were surface sterilised in 5% sodium hypochlorite solution (NaOCl) for 5 min., washed in two changes of sterile distilled water and plated on potato dextrose agar (PDA). The resulting cultures were sub-cultured onto fresh

PDA plates until pure cultures were obtained. All cultures of the isolates were subcultured onto and maintained on PDA throughout this study unless otherwise stated.

Effect of Environmental Factors on Growth, Sporulation and Cultural Characteristics of Colletotrichum gloeosporioides *isolates*

For this study and subsequent studies, the four *Colletotrichum* isolates as shown in Table 1 were used as the test fungi. To study the effect of temperature, each PDA plate was centrally inoculated with 5mm diameter fungus plug taken from the advancing margin of a 5-day old culture and incubated in the dark at 20, 25, 30 and 35°C for five days.

TABLE 1
Isolates of *Colletotrichum gloeosporioides* from infected cocoa leaf and pod associated with leaf spot and pod rot disease

Isolate	Source
Sh	cocoa leaf with shot hole symptom
Lb	cocoa leaf with blight symptom
Is	cocoa leaf with irregular spot symptom
Pr	cocoa pod with pod rot symptom

The effect of culture media on the growth, sporulation and cultural characteristics were determined by centrally inoculating PDA, CDA (Czapek Dox Agar), MEA (Malt Extract Agar), OMA (Oat Meal Agar), Cooks (Cooks medium) and CLEA (Cocoa Leaf Extract Agar) plates with a 5mm diameter fungus plug taken from the advancing margin of a five-day old culture and incubated at 30°C in the dark for five days.

Each treatment was replicated four times. Growth measurements, degree of sporulation and cultural characteristics were assessed at the end of the experiment. Mycelial growth was assessed by taking the average of the two perpendicular distances across the centre of the colony. Spore concentration was determined with the aid of a Neubauer haemocytometer.

Infectivity Studies

Cocoa seedlings of mixed hybrid of 2, 3 and 4-week old were inoculated with spore suspension of 10^6 conidia/ml until run-off. The spore suspension was prepared by flooding a five-day old culture; three drops of Tween 80 were then added to the resultant spore suspension before spraying. Sterile distilled water was used as the control. Seedlings were maintained in a moist environment for infection to occur. The experiment was replicated ten times, arranged in randomized complete block design (RCBD) with each replication consisting of a single seedling. Assessment of percentage leaf area infected was conducted on the tenth day.

Infectivity studies were also carried out on detached cocoa pod; Cherelle (25-30 mm long), young pod (40-50 mm long) and green mature pod (85-100 mm long) of mixed hybrid which were surface sterilised with 5% NaOCl and rinsed with sterilised water. Pods were inoculated directly or injured with a 0.5mm diameter sterilised inoculating needle prior to inoculation. Pods were spot inoculated with 10µl of spore suspension of 10^6 conidia/ml. The inoculated pods were incubated in a moist chamber at 30°C with 98% relative humidity. Ten pods of each size were used for each treatment. The control treatments were inoculated with sterile distilled water. Lesions were assessed on the tenth day by taking the average of the two perpendicular distance across the centre of the lesion.

Data were statistically analysed and the difference between individual means was tested using Duncan Multiple Range Test (DMRT).

RESULTS

The Pathogen

Isolation made from the three types of foliar symptoms viz: shot-hole, leaf blight and irregular spot and a pod rot symptom consistently yielded the fungus *Colletotrichum*. Cultural studies on the pure cultures obtained showed that the cultures isolated from infected tissues showing the same type of symptom, were similar to one other; however, they were different from those isolated from other types of symptom. Results as shown in Table 2 suggest the identity of the four cultures was close to that of *C. gloeosporioides* (Penz.) Sacc. The identification was confirmed by IMI, Kew, England. No other distinct characteristics were noted which could facilitate strain differentiation within the species.

TABLE 2
Cultural and morphological characteristics of 5-Day old *C. gloeosporioides* isolates from cocoa grown on PDA

Isolate	Cultural characteristics	Morphological characteristics
Sh	Colony appeared white and gradually turned greyish salmon in colour as the culture grew older. Aerial mycelium slightly flocculose with orange conidial pustules evident at the centre of the colony. Reverse of colony appeared smoky grey in colour.	Conidia cylindrical with obtuse ends, hyaline, aseptate, uninucleate, 5-22 µm x 2-6 µm, formed in setose or globose acervuli or on solitary phialides on mycelium. Acervulus round to elongated to irregular 60-240 µm in diameter. Setae sparse to profuse, dark brown to black, straight to slightly curved, 1-4 septate, swollen at the base and tapering towards the apex, 50-170 µm long.
Lb	Colony appeared white and gradually turned olivaceous grey in colour as the culture grew older. Aerial mycelium flocculose with orange conidial pustules apparent at the centre of the colony. Reverse of colony appeared smoky grey in colour.	Conidia cylindrical with obtuse ends, hyaline, aeptate, uninucleate, 4-24 µm x 2.5 µm, formed in setose or globose acervuli or on solitary phialides on mycelium. Acervulus round to elongated to irregular, 70-250 µm in diameter. Setae sparse to profuse, dark brown to black, straight to slightly curved, 1-4 septate, swollen at the base and tapering towards the apex, 50-80 µm long.
Is	Colony appeared white and gradually turned greyish white as the culture grew older. Aerial mycelium slightly flocculose with orange conidial pustules evident at the centre of the colony. Reverse of colony appeared smoky grey in colour.	Conidia cylindrical with obtuse ends, hyaline, aseptate, uninucleate, 4-23 µm x 2-6 µm, formed in setose or globose acervuli or on solitary phialides on mycelium. Avervulus round to elongated to irregular 65-190 µm in diameter. Setae were sparse to profuse, dark brown to black, straight to slightly curved, 1-4 septate, swollen at base and tapering towards the apex, 70-165 µm long.
Pr	Colony appeared smoky grey in colour with thick floccose aerial mycelium and orange conidial pustules at the centre. Reverse of colony appeared in the form of distinct olivaceous grey zonation alternated with rosy buff zonation.	Conidia cylindrical with obtuse ends, hyaline, aseptate, uninucleate, 4-22 µm x 2-5 µm, formed in setose or globose acervuli or on solitary phialides on mycelium. Acervulus round to elongated to irregular, 80-230 µm in diameter. Setae sparse to profuse, dark brown to black, straight to slightly curved, 1-4 septate, swollen at the base and tapering towards the apex, 80-170 µm long.

Effect of Environmental Factors on Growth and Sporulation of Colletotrichum gloeosporioides Isolates

Studies on the effect of temperature on the mycelial growth and sporulation (Fig. 1A & 1B) of the four *Colletotrichum* isolates proved to be significant at P = 0.05. All the four isolates were found to grow better at 30°C over an incubation period of 5 days Sh and Lb isolate attained a mycelial growth of 51 mm. in diameter Is isolate 37 mm. and Pr isolate, 30mm. The fungi were also found to sporulate better at 30°C with the highest number of spores being harvested from Is isolate ($9x10^6$ spores/ml), followed by Pr isolate ($8x10^6$ spores/ml), Sh isolate ($2x10^6$ spores/ml) and Lb isolate ($1x10^6$ spores/ml).

Growth media influence the growth rate and sporulation of *Colletotrichum* isolates. However, the variation in growth and sporulation was insignificant at P = 0.05. CLEA was noted as the best medium for mycelial growth followed by OMA, Cook's, PDA, MEA and CDA. On the other hand, CLEA, failed to maintain favourable

sporulation. PDA was the best sporulation medium followed by CDA, OMA, MEA, Cook's and CLEA.

Infectivity Studies

Each isolate could produce more than one type of symptom depending on the leaf age and the

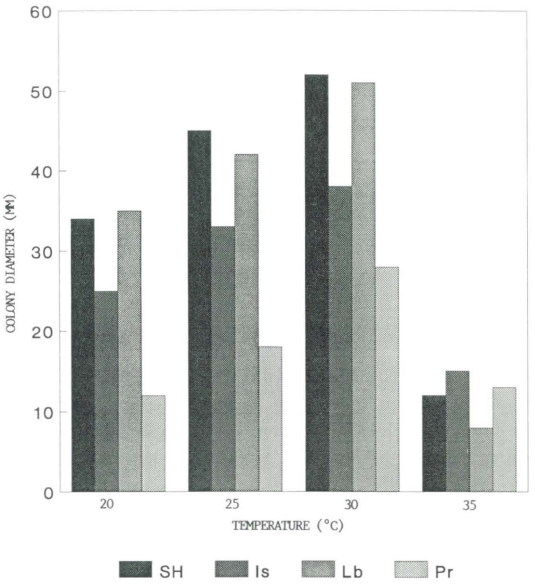

Fig. 1A: Effect of Temperature on the Linear Growth of C. gloeosporioides *Isolates from Cocoa on PDA* ($L.SD_{0.05}$= *8.47)*

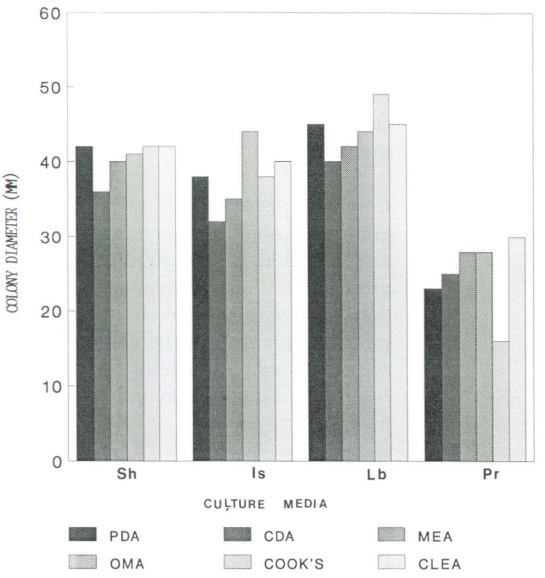

Fig. 2A: Effect of Culture Media on the Linear Growth of C. gloeosporioides *Isolates from Cocoa on PDA*

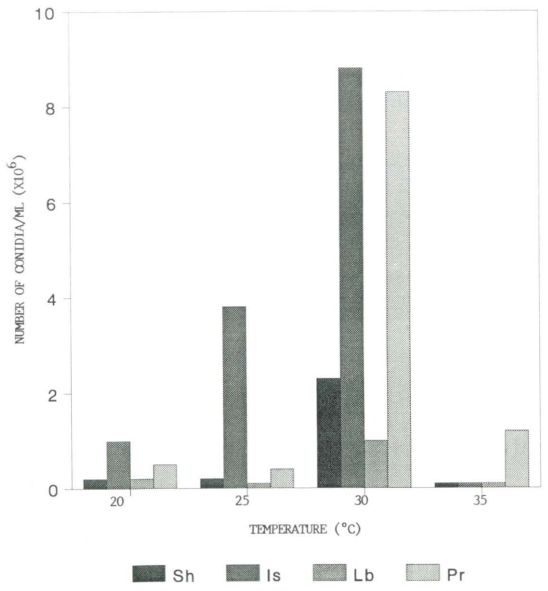

Fig. 1B: Effect of Temperature on Sporulation of C. gloeosporioides *Isolates from Cocoa on PDA* ($L.SD_{0.05}$= *8.47)*

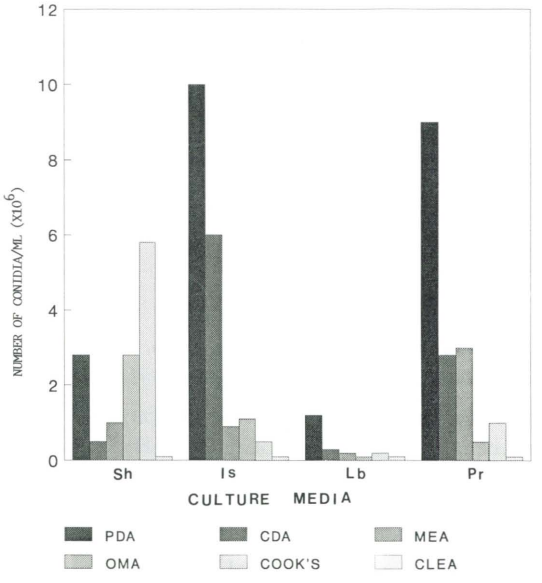

Fig. 2B: Effect of Culture Media on the Sporulation of C. gloeosporioides *Isolates from Cocoa on PDA*

extent of the infection. Blight and shot-hole symptoms were more frequently observed on younger leaves although they were also seen on older leaves. Irregular spot symptom, however, dominated on the older leaves with blight symptoms being occasionally observed. The first evidence of infection was noted after four days of incubation. The symptoms caused by the various isolates of *Colletotrichum* were undistinguishable at the initial stage of infection. The lesion first appeared as minute yellowish specks, and later discernible as circular reddish brown lesions with a chlorotic halo. As the infection progressed, three clearly distinguishable foliage symptoms viz, shot-hole, irregular spot and blight were apparent on the affected seedlings. The results presented in Table 3 indicate that Pr isolate was the most virulent one tested on the cocoa seedlings, recording a mean percentage leaf area infection of 2.2%, 12.2% and 11% on 2-week, 3-week and 4-week old cocoa seedlings respectively. Isolate Is was the least pathogenic, exhibiting a mean percentage leaf area infected of 1.1%, 6.5% and 6.0% on 2-week, 3-week and 4-week old seedlings respectively.

Lesions incited by the various cocoa isolates of *Colletotrichum* on detached injured pods are shown in Table 4. The severity of infection on cherelle was significantly different from that of green mature pod. Isolate Pr was noted to be the most pathogenic strain followed by Sh, Is and Lb. Infection was first observed on the inoculation spot 5 days after incubation in the form of a small brownish round spot with a yellow halo. The affected area later became darker and formed a depressed lesion, followed by the production of greyish white mycelium with pink coloured masses of conidia. Lesions caused by the various isolates of *Colletotrichum* appeared identical and reisolation from pod lesion yielded only the isolate with which the particular pod was inoculated. No infection was noted in any of the non-wounded inoculated pods.

TABLE 3
Severity of infection incited by *Colletotrichum gloeosporioides* isolates on cocoa seedlings of different ages

Treatment	Mean percentage leaf area infected*				
Seedling Age	Is	Lb	Sh	Pr	Control
2 weeks	1.1[a]	1.7[a]	1.8[a]	2.2[a]	0[a]
3 weeks	6.5[b]	8.8[b]	7.4[b]	12.2[b]	0[a]
4 weeks	6.0[b]	8.0[b]	6.8[b]	11.0[b]	0[a]

* Any two means within the column followed by the same letter are not significantly different at 5% level based on Duncan Multiple Range Test.

TABLE 4
Lesion development caused by *Colletotrichum gloeosporioides* isolates on injured detached cocoa pods of various sizes

Treatment	Lesion size (mm)*				
Pod size	Is	Lb	Sh	Pr	Control
Cherelled	4.8[a]	3.5[a]	10.4[a]	13.0[a]	0[a]
Young pod	4.7[a]	0[b]	8.3[b]	12.5[a]	0[a]
Green mature pod	0[b]	0[b]	0[c]	7.5[b]	0[c]

* Any two means within the column followed by the same letter are not significantly different at 5% level based on Duncan Multiple Range Test.

DISCUSSION

Isolation of the disease pathogen from the respective types of foliar symptoms viz., shot-hole, leaf blight and irregular spot and pod rot yielded *Colletotrichum* isolates. These isolates were identified as *Colletotrichum gloeosporioides* (Penz.) Sacc. and subsequently confirmed by the International Mycological Institute. Cultural and morphological studies showed no distinct differences in characteristics among the *C. gloeosporioides* isolates which could facilitate strain differentiation within the species, except for slight variation in culture colour and consistency of the mycelium. All the *C. gloeosporioides* isolates produced cylindrical conidia with obtuse ends, hyaline, aseptate, uninucleate and measured 4-24µm x 2-6µm which were formed in setose or globose acervuli. The shape of the acervuli ranged from round to elongated to irregular and measured 60-250µm in diameter. Setae were sparse to profuse, dark brown to black, straight to slightly curved, 1-4 septate, swollen at the base and tapering towards the apex.

Studies on the species of *Colletotrichum* elsewhere have shown them to be very variable in their morphological (Arx, 1970) and cultural

characteristics (Stoneman, 1898; Shear and Wood, 1913; Burger, 1921). Mohanan (1983) was able to classify the isolates of *C. gloeosporioides* from cocoa associated with irregular spot, blight and shot-hole symptom into white, dark and light types. Dakwa and Danquah (1978) also observed distinct variation in morphological and cultural characteristics among the isolates of *C. gloeosporioides* which cause leaf blight of cocoa in Ghana.

Studies on the effect of temperature on mycelial growth and sporulation of *C. gloeosporioides* isolates proved to be significant at P = 0.05. The isolates were found to grow and sporulate better at 30°C on PDA. Similar observations were made by Quimio (1974) and Scattar and Malik (1938) on *C. gloeosporioides* from mango, and Lii (1972) on *C. gloeosporioides* from guava. Earlier work by Wastie and Shanker (1970) and Muthappa (1971) were confirmed by our studies that growth media could influence the growth rate and sporulation of *C. gloeosporioides*. However, the effects were insignificant (P = 0.05). CLEA was observed to be the best medium for mycelial growth followed by OMA, COOK'S, PDA, MEA and CDA. In our study, however, CLEA failed to maintain its status in favouring sporulation; we noted that PDA was the best sporulation medium followed by CDA, OMA, MEA, COOK'S and CLEA. Radziah (1985) claimed that PDA and CDA favour sporulation while working on *C. gloeosporioides* from rubber.

Results obtained from our infection studies reveal that *C. gloeosporioides* could infect both cocoa leaves and pods. Wilting of cherelles and young pods were generally considered to be due to physiological factors, but recent observations by Mohanan and Kaveriappa (1983) showed that a considerable percentage of pod rot of cherelles and young pods was due to *Colletotrichum* infection. Similar observations were reported by Bailey (1966) from Nigeria and Reddy and Mohanan (1976) from India. Mohanan and Kaveriappa (1983) in their studies on the symptomatology of *Colletotrichum* disease of cocoa reported that the occurrence of three different types of symptoms on the foliage of cocoa plant caused by *C. gloeosporioides* could be attributed to the existence of different varieties of the same species or pathological strain. However, in our studies, we found that each isolate could produce more than one type of symptom depending on the leaf age and the extent of infection. Wastie and Shankar (1970) claimed that apart from climatic factors, leaf age has an influence on the severity of *Colletotrichum* infection. Blight and shot-hole symptoms were more prominent on younger leaves although they were also spotted on older leaves. On the other hand, irregular spot symptoms dominated on the older leaves with blight symptom being occasionally observed. Dakwa and Danquah (1978) reported similar observations. Spotting of older leaves caused little damage; however, infection on newly formed young leaves could result in impairment of the functional photosynthesis (Sarma and Nambiar, 1976) and leaf fall, producing bare tips which could subsequently be invaded by *Botryodiplodia theobromae* Pat. (Shell Chemicals Technical Bulletin).

Laboratory studies with detached cocoa pods showed that only injured cocoa pods were liable to infection by *C. gloeosporioides* conidia and that cherelles and young pods were more susceptible than green mature pods. Although pathogenicity tests were only carried out in the laboratory, this information suggests a possible mode of penetration for field infection. Various factors have been linked to the initiation of spore germination and disease spread. Mohanan (1983) suggested that free water available on leaf surface could facilitate conidial germination and infection. Sarma and Nambiar (1976) reported that although shot-hole incidence was found throughout the year in Kasaragod district of Kerala, the intensity was higher when a temperature range of 19 - 33°C and relative humidity of 77 - 98% prevailed. On the other hand, Dakwa and Danquah (1978) were of the opinion that the high incidence of leaf blight in Ghana could be attributed to the availability of tender leaves coupled with the high relative humidity and moisture on leaf surface. Although *Colletotrichum* disease of cocoa is not very serious at present in this country, its wide distribution and occurrence makes it necessary for effective control measures to be taken to prevent the fungus from attaining epiphytotic proportions as has happened in Ghana.

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