MORPHOLOGY AND CULTURAL VARIATION AMONG *COLLETOTRICHUM* ISOLATES OBTAINED FROM TROPICAL FOREST NURSERIES

Maziah Zakaria

Forest Research Institute Malaysia (FRIM), Kepong, 52109 Kuala Lumpur, Malaysia

&

John A. Bailey

Department of Agricultural Sciences, University of Bristol, Long Ashton Research Station, Long Ashton, Bristol BS18 9AF, United Kingdom

MAZIAH, Z. & BAILEY, J. A. 2000. **Morphology and cultural variation among** *Colletotrichum* **isolates obtained from tropical forest nurseries.** Surveys of forest nurseries in Malaysia have shown that *Colletotrichum* is one of the major fungi associated with seedling diseases. Morphological and cultural characteristics of 12 *Colletotrichum* isolates from several forest nurseries in Peninsular Malaysia and an isolate from Guatemala were compared. Results from the preliminary morphological studies suggest that most of the isolates produced ovoid/straight conidia with rounded apices typical of *C. gloeosporioides.* The exception was isolate 689 (ex *Gliricidia sepium,* from Guatemala) which produced ovoid conidia with pointed apices. Morphological analysis of the other isolates illustrated that most of them showed variation in their conidial size, appressorial shape and size, suggesting the existence of different forms of *C. gloeosporioides.* The results also revealed distinct appressorial shapes. Some produced globose appressoria, whilst in others appressoria were lobed. These features were reliable and reproducible characteristics. All isolates became septate on germination, suggesting that none of them are members of the *C. orbiculare* group. Other criteria such as culture characteristics, the presence and dimensions of setae, colour of conidial mass, growth rate and number of conidia nuclei were either too variable or too similar to make them useful taxonomic criteria. In addition to culture characteristic studies, lectin cytochemistry study, which was also conducted but not reported in this paper, was found to be not significant in distinguishing the forest tree isolates. From this study it was found that morphological study alone has many limitations in trying to distinguish species of *Colletotrichum.*

Key words: *Colletotrichum gloeosporioides* - cultural variation - morphology - taxonomy - forest tree - tropical forest

MAZIAH, Z. & BAILEY, J. A. 2000. **Variasi morfologi dan kultur di kalangan isolat** *Colletotrichum* **dari tapak semaian pokok hutan tropika.** Daripada beberapa survei yang dilakukan di tapak semaian pokok hutan di Malaysia didapati *Colletotrichum* merupakan salah satu kulat utama yang berasosiasi dengan penyakit anak benih. Morfologi dan ciri kultur 12 isolat *Colletotrichum* dari beberapa buah tapak semaian hutan di Semenanjung Malaysia dan satu isolat dari Guatemala telah dibandingkan. Keputusan

daripada kajian morfologi awal menunjukkan kebanyakan isolat menghasilkan konidia yang lurus/bujur dengan hujung yang bulat seperti yang biasa terdapat pada *C. gloeosporioides*, kecuali isolat 689 (ex *Gliricidia sepium*, dari Guatemala) yang menghasilkan konidia bujur dengan hujung yang tajam. Analisis morfologi ke atas kebanyakan isolat yang lain menunjukkan terdapat variasi dari segi ciri-ciri saiz konidia serta bentuk dan saiz apresoria, ini mencadangkan kehadiran beberapa bentuk *G. gloeosporioides*. Keputusan kajian juga menunjukkan terdapat perbezaan yang nyata dalam bentuk apresoria. Sesetengah isolat menghasilkan apresoria berbentuk globos sementara yang lainnya berbentuk 'lobed'. Ciri-ciri ini boleh digunakan dengan berkesan untuk perbandingan dan ia boleh dihasilkan dari semasa ke semasa. Semua isolat membentuk septa semasa percambahan, ini menandakan tiada antaranya daripada kumpulan *C. orbiculare* yang tidak membentuk septa semasa percambahan. Ciri-ciri lain seperti ciri kultur, kehadiran dan dimensi seta, warna 'mass' konidia, kadar pertumbuhan dan bilangan nuklei pada konidia adalah terlalu berbeza atau terlalu sama untuk dijadikan ciri perbandingan taksonomi yang berguna. Di samping kajian ciri kultur, kajian lektin sitokimia juga telah dilakukan tetapi keputusan tidak dilaporkan di sini kerana ia tidak memuaskan untuk membezakan isolat dari pokok hutan. Daripada kajian yang dijalankan boleh disimpulkan bahawa kajian ciri morfologi sahaja tidak memadai untuk membezakan antara spesies-spesies *Colletotrichum* kerana terdapat banyak batasannya.

Introduction

Tropical forests are disappearing in most countries. In Southeast Asia their disappearance is largely due to clearing for agriculture production, industrial development and excessive logging. One of these countries is Malaysia, once an exporter of tropical timber, and projected to become a timber importer by the year 2000. In order to reduce its reliance on indigenous forest species, the government has adopted a strategy of establishing forest plantations of fast-growing indigenous and exotic species. The establishment of large-scale forest plantations has led to the establishment of extensive forest nurseries. Seedling diseases represent a serious threat, especially since much of the required seedling propagation is carried out in nurseries sited within afforested areas, where they are surrounded by trees. These trees may provide a continuous source of inoculum.

Disease surveys conducted by the authors revealed the regular occurrence of *Colletotrichum* species in forest nurseries. They occur on a wide range of different host species and cause a variety of symptoms especially on the foliage (Maziah 1995). They are present throughout the year and are more prevalent during or after the rainy seasons.

The occurrence of *Colletotrichum* diseases on perennial crops is very well documented (Waller 1992). However, reports of their occurrence on forest trees are still scarce. Most available reports are on those diseases occurring in nurseries. Those occurring in plantations and natural forests are not being researched as their long term impact on the final crop is considered minor, and also because control of diseases at this stage is neither feasible nor economical.

In the past, the most important forest tree disease in Malaysia was brown needle disease of pines caused by *C. gloeosporioides* (Freezailah & Low 1968). The pathogen attacked all species of pines grown in the nursery, but it appeared to be more virulent on *P. caribaea* than on other *Pinus* species (Fielding 1971). The causal organism, *C. gloeosporioides,* is probably the most widespread and heterogeneous of all *Colletotrichum* species. It exhibits considerable morphological and pathogenic variation, and has proved difficult to classify using morphological characters (Sutton 1980). Over 600 synonyms have been cited for this species alone (Sutton 1992).

Simple morphological characteristics, such as conidial dimensions, appressorial dimensions, presence or absence of setae and perithecia and also growth rate have been widely used as taxonomic criteria within the genus *Colletotrichum* in the past (Sutton 1992). The present studies are aimed at assessing the values of such criteria in distinguishing the isolates from forest nurseries in Malaysia.

Materials and methods

In the present study, the sources of isolates used are listed in Table 1. Diseased materials were collected from several forest nurseries in Peninsular Malaysia. Tissues bearing symptoms were excised and surface-sterilised with sodium hypochlorite (0.35%) for 2–4 min depending on the thickness of the tissues, placed on potato dextrose agar (PDA) and incubated for 48 h at 30 °C. Thirteen *Colletotrichum* isolates (630, 634, 635, 640, 645, 657, 659, 660, 662, 664, 665, 674 and 689) were identified and selected for detailed morphological studies.

Table 1. Isolate number, hosts and symptoms of 13 chosen
Colletotrichum isolates

Isolate No.	Original host	Disease symptoms
630	*Acacia mangium*	Leaf spots and lesion
634	*Hevea brasiliensis*	Leaf spots
635	*Chrysalidocarpus lutescens*	Leaf spots
640	*Schizostachym branchycladium*	Leaf spots
645	*Magnolia malayana*	Leaf lesions
657	*Calamus manan A*	Leaf spots and lesions
659	*Calamus manan B*	Leaf spots and lesions
660	*Pterocarpus indicus A*	Leaf spots and lesions
662	*P. indicus B*	Leaf spots and lesions
664	*P. indica C*	Leaf spots and lesions
665	*P. indica D*	Leaf spots and lesions
674	*Schoutenia accrescens*	Leaf spots
689	*Gliricida sepium*	Leaf spots

Note: Except for isolate 689 from Guatemala, the rest of the isolates were collected from Malaysia.

Preparation of conidial suspension

All the isolates were cultured on Mathur's agar medium (CM) (Mathur *et al.* 1949). Conidia were rinsed from the surface of a 7-day-old culture growing in conical flasks and filtered through three layers of muslin to remove mycelial fragments. It was then washed twice by centrifugation at 2000 r.p.m. for 3 min and re-suspended in sterile distilled water to remove the mucilaginous material encasing the conidia, thus improving the rate of germination. The concentration was adjusted to 5×10^5 conidia ml^{-1} using a haemacytometer.

Assessment of conidial germination and differentiation

Drops of the conidial suspension were placed in multi-welled microscope slides. Conidial shape and size were observed with bright light microscopy. Size was determined by measuring the length and width of 50 randomly-chosen conidia using a micrometer.

In addition, the multi-welled slides were placed in a transparent box that had been lined with moist tissue to prevent dehydration, and incubated at 25 °C for 24 to 48 h. The formation of septa and dimensions of appressoria were determined by examining 50 randomly chosen conidia.

The existence of septa was confirmed by staining the samples with a fluorescent dye, as some septa were not clearly visible in fresh material. The fluorescent dye used was Calcofluor white M2R (Sigma F6259). A drop of Calcofluor solution (0.01 % w/v) was added to each well and after 2-5 min, fluorescent walls including septa were easily recognisable under u.v.- epifluorescence microscopy.

Assessment of culture morphology and growth characteristics

Culture morphology and growth rate, as well as presence of setae and perithecia, were determined by growing the isolates on various media, namely potato dextrose agar (PDA), corn meal agar (CMA), Mathur's agar medium (CM) and oat meal agar (OMA). A plug of mycelium was placed at the centre of agar plates and incubated at 25 °C under near ultraviolet light (Philips TL40w/08). For growth-rate studies, the plates were marked so that two measurements of diameter could be taken at right angles. The colony diameters were measured daily for five days and the maximum growth rate, usually between the second and fifth days, was calculated.

Mycelial colour and appearance were determined by examining the cultures against white background after 9-day growth. Sporulation was determined by centrifuging conidial suspension from three flasks of each medium (PDA, CMA, CM and OMA) and comparing the amount of conidial mass deposited in the centrifuge tubes.

The presence of setae was determined by examining 7- to 14-day-old cultures. Their abundance on different media was recorded by observing a minimum of 20 setae in each isolate. The lengths and widths of their bases were measured.

For assessment of perithecia production, cultures were examined after one week of incubation and every three days thereafter for the next five weeks. Plates were sealed with Parafilm during incubation to prevent dehydration.

Assessment of the number of nuclei present in conidia

Nuclear number was observed using DAPI (4',6-diamidino-2-phenylindole). This experiment was undertaken to determine the number of nuclei present in each isolate and whether this criterion could be used to distinguish the isolates.

Clean multi-welled slides were coated with 0.1 % w/v poly-L-lysine hydrobromide (M_r = 15 000–30 000, Sigma, Product No. P 7890), and left to dry. Conidial suspensions were centrifuged and resuspended in 1% glutaraldehyde for 30 min, then rinsed three times with phosphate buffered saline. Drops of conidial suspension were placed on the coated slide and left to dry. Droplets of aqueous DAPI (1 µg ml^{-1}) were placed over the conidia. After 5 to 10 min, the slides were rinsed by placing them in water in a Coplin jar for 5 min. Observations were done using u.v.-epifluorescence microscopy with the appropriate filter set. When necessary, toluidine blue (0.01%) was added to the well to suppress auto-fluorescence.

Results

Conidial morphology

Conidia were observed to be produced in acervuli and by mycelia. All isolates produced hyaline, unicellular, smooth walled, ovoid conidia with length to width ratio of about 3 : 1. Generally, conidial shape of most isolates looked very similar. Conidia of most isolates had rounded apices, except those of isolate 689, which had pointed apices (Table 2).

Table 2. Morphological data of the 13 *Colletotrichum* isolates from forest trees

Isolate	Conidial shape and apex	Mean conidial length (µm)	Mean conidial width (µm)	Appressorial shape	Perithecia	Setae	Growth rate (mm d⁻¹)
630	Ovoid, obtuse	12.8 ± 0.67	5.0 ± 0.34	Lobed	Absent	Present	10.9±0.58
634	Ovoid, obtuse	17.9 ± 2.61	5.0 ± 0.40	Globose, subglobose	Sterile	Present	13.6±0.29
635	Ovoid, obtuse	12.1 ± 4.48	5.0 ± 0.49	Globose, subglobose	Absent	Present	4.3 ± 2.08
640	Ovoid, obtuse	15.7 ± 2.49	5.0 ± 0.49	Subglobose, lobed	Present	Present	12.1±0.29
645	Ovoid, obtuse	18.4 ± 2.42	5.5 ± 0.66	Globose, subglobose	Absent	Absent	11.8±2.52
657	Ovoid, obtuse	16.9 ± 1.13	4.9 ± 0.35	Globose, subglobose	Absent	Present	11.8±1.53
659	Ovoid, obtuse	12.7 ± 0.48	7.5 ± 0.14	Subglobose, lobed	Absent	Absent	9.7±1.44
660	Ovoid, obtuse	17.7 ± 1.78	5.2 ± 0.55	Lobed	Present	Present	14.2±0.29
662	Ovoid, obtuse	22.2 ± 3.61	7.8 ± 0.55	Lobed	Absent	Present	9.2 ± 0.50
664	Ovoid, obtuse	17.5 ± 1.22	5.0 ± 0.55	Absent	Sterile	Absent	11.1±0.76
665	Ovoid, obtuse	18.4 ± 1.24	5.0 ± 0.30	Subglobose, lobed	Sterile	Present	11.5±1.32
674	Ovoid, obtuse	39.5 ± 2.65	10.7 ± 0.66	Lobed	Absent	Present	8.9±1.76
689	Ovoid, acute	13.5 ± 2.18	4.6 ± 0.51	Globose, subglobose	Absent	Absent	6.0±0.50

Results summarised in Table 2 demonstrated considerable variation in conidial dimensions, particularly the width. Variability of conidial length within the isolates was greatest in 635. Between isolates, the ratio of the length of the shortest (659) to the longest (674) was also 1 : 3. In terms of width, three isolates (659, 664, and 674) had widths greater than 6 μm. The ratio of the narrowest (689) to the broadest (674) isolate was about 1:2.

Conidia of most isolates were in the range of 12–20 μm × 4.5–8 μm. Isolate 674 was very distinct because of its size (39.5 × 10.7 μm). Such conidia were more than 17 μm larger than that of the next biggest (22.2 × 7.8 μm). Isolate 630 was distinct because of the short (12.8–13.5 μm) conidia. No other groupings were identified.

Conidial germination and appressorial formation

Prior to germination, conidia were observed to swell slightly and the cytoplasm changed from finely granular to coarsely granular. Upon germination, a septum or occasionally two septa formed in the middle of the conidia of all the isolates examined in this study. This was associated with the formation of a small protrusion of a germ-tube at the point of emergence, usually at the end of the conidium. At the tip, the germ-tube swelled as the cytoplasm moved into the developing appressorium. At the same time, a wall was formed, separating the appressorium from the germ-tube. Apart from ending with appressoria, the germ-tubes of a few isolates were observed to produce another conidium resulting in a population of a parent conidium interconnected to several other conidia; this process is known as microcyclic conidiation.

Appressorial morphology

Generally, appressoria were produced by germ-tubes or hyphae. Those produced by germ-tubes were usually terminal, formed either immediately adjacent to the conidia (sessile) or at the end of a long germ-tube. In addition, germ-tubes sometimes branched and several appressoria formed on the branched ends. Although most isolates in this study produced appressoria on glass surfaces, isolate 664 failed to do so. Appressoria were single-celled. Initially they were hyaline, but rapidly became melanised and at maturity, produced a single central germ-pore. Appressoria were produced within 24 h by most isolates, except isolate 634 which produced them after 36–48 h. During appressorium formation, most conidial cytoplasm moved into the developing appressorium leaving an empty conidium.

Variations were seen in appressorial dimensions, shape and colour. The shape of the appressoria can be categorised into four types, namely globose, globose and subglobose, subglobose and lobed, and strongly lobed. Of the 13 isolates, only one (664) did not produce appressoria, seven (630, 634, 635, 645, 657, 665 and 689) bore appressoria with predominantly smooth surfaces, and two (640 and 659) bore appressoria with a higher percentage of slightly lobed than smooth surfaces. Three isolates (660, 662 and 674) were distinct because they bore a high percentage of obviously lobed appressoria (Table 3).

Table 3. Appressorial morphology of the 13 *Colletotrichum* isolates

Isolate	Wall type (%)		
	Smooth	Slightly lobed	Obviously lobed
630	98	2	0
634	88	12	0
635	90	10	0
640	44	56	0
645	90	10	0
657	85	15	0
659	22	78	0
660	0	10	90
662	0	43	57
664	-	-	-
665	68	32	0
674	0	0	100
689	97	3	0

Note: observations were done five days after placing conidial droplets on glass slides.

On the whole, the results showed that appressorial shape is a consistent feature within an isolate but their size is more variable. Generally, appressorial size is proportional to the conidial size of the isolates. The colour of appressoria ranged from light to very dark brown. Globose and subglobose appressoria were usually lighter brown than lobed appressoria. Some conidia germinated but did not produce appressoria immediately. If these were left on the slide for 7–14 days, appressoria were formed on hyphal ends. These appressoria were extremely variable and complex in shape.

Setal morphology

Setae were found to be present in 9 (630, 634, 635, 640, 657, 660, 662, 665 and 674) of the 13 isolates (Table 2). Most setae were formed in acervuli but occasionally they were also formed on individual hyphae. Setae were thick-walled, melanised and multi-septate. In some isolates setae were abundant, but in others they were sparse. The abundance and the length of setae contributed to the degree of darkness of the colonies.

Variations in setal size, shape and number of septation were observed. Their dimensions varied considerably from one isolate to another. Setae of most isolates were cylindrical with rounded apices. In general, longer setae had more septa than the shorter ones with the number of septa ranging from 1 to 3. For most of the isolates, setae length ranged from 52.80 μm (630) to 79.84 μm (640) (Table 4). Setal lengths of three isolates (660, 662 and 674) were relatively longer than the rest.

Table 4. Setae dimensions of the 13 *Colletotrichum* isolates 66

Isolate	Length of setae (μm)				Width at base (μm)			
	Mean	Std Dev.	Max.	Min.	Mean	Std Dev.	Max.	Min.
630	52.80	6.79	60.80	46.40	6.72	1.09	8.00	5.60
640	79.84	14.96	112.00	64.00	4.16	0.83	5.60	2.88
645	58.72	9.72	72.00	41.60	4.03	0.72	4.80	3.20
657	71.84	20.44	107.20	48.00	3.52	0.41	4.00	3.20
660	96.00	18.64	126.40	67.20	3.92	0.72	4.80	2.88
662	130.30	36.87	224.00	96.00	5.44	1.45	8.00	3.20
665	62.34	6.49	75.20	49.00	3.68	0.67	4.80	3.20
674	116.80	24.71	163.20	80.00	8.91	0.74	9.60	8.80

Note: Isolates 634, 569, 664, 665 and 689 did not produce setae.

Production of conidia at the tip of setae was also observed in a few isolates. Generally, setae which produced conidia were distinguishable from sterile setae by their hyaline and blunt apices compared to the brown pointed apices of the sterile setae.

Perithecial morphology

Only two isolates (640 and 660) produced fertile perithecia, with abundant ascospores. The rest were either sterile or did not produced perithecia at all (Table 2). Generally, fertile perithecia were formed in clumps, whereas the sterile ones were scattered over the whole surface. Perithecia were rounded or flask-shaped, pigmented and with well-developed ostioles. Within the perithecia were cylindrical, thin-walled asci, each containing eight ascospores. The ascospores were fusiform (curved), hyaline and aseptate. In all isolates that produced perithecia, ascospores were easily distinguished from their conidia in both shape and size. The shape of ascospores varied only slightly between the two isolates.

Growth rate of cultures

The mean colony growth for isolates on PDA during a three-day (day 2–5) period is shown in Table 2. The rate of growth of isolates ranged from 4.30 to 14.20 mm day^{-1}. The rate of growth of isolate 660 (14.20 mm day^{-1}) was the fastest, followed by isolate 644 (14.00 mm day^{-1}). By contrast, the slowest growing culture was isolate 635 (4.27 mm day^{-1}) followed by isolates 689 (6.00 mm day^{-1}).

Cultural characteristics

Studies on cultural characteristics aimed at identifying simple cultural criteria that could differentiate between isolates were conducted. The growth media used in these studies were PDA, CMA, CM and OMA.

Mycelial growth

Variation in mycelial growth occurred between media. Mycelial growth was very profuse on PDA, moderate on OMA and CMA and least on CM. Sectoring was observed to occur in some isolates on PDA, but rarely on the other media. For all isolates, acervuli production was extensive on OMA and CM, but more moderate on PDA and CMA.

Mycelial colour and appearance

The appearance of colonies in terms of mycelium colour and structure on PDA is described in Table 5. PDA was selected because this medium has been used most frequently by other workers in comparing cultural appearance of *Colletotrichum*. Generally these characteristics showed a gradual tendency from white to grey mycelium. No culture was quantitatively different.

Table 5. Culture characteristics of the 13 *Colletotrichum* isolates on PDA

Isolate	Appearance of culture
630	Mycelium velvety, even. Colony white-beige; orange spore masses. Reverse light orange to dark brown in centre.
634	Mycelium velvety, slightly flattened at the centre. Colony white to light grey. Reverse white to light olive.
635	Mycelium thick, fluffy. Colony dark grey-olive. Reverse dark olive-black.
640	Mycelium woolly but slightly flattened. Colony greyish-white to olive. Reverse white with greyish-orange centre.
645	Mycelium dense and floccose. Colony grey, pinkish-orange. Reverse orange-pink with mixture of grey.
657	Mycelium dense but slightly downy. Colony light beige. Reverse beige.
659	Mycelium even and floccose. Colony light to dark grey. Reverse dark grey.
660	Mycelium thin and woolly. Colony greyish-brown. Reverse black at the centre and greyish-brown at the margin.
662	Mycelium moderate, woolly. Colony evenly dark olive. Reverse dark olive-grey.
664	Mycelium thin, even, flattened. Colony light greyish-orange. Reverse greyish, with orange centre.
665	Mycelium woolly. Colony white to grey. Reverse beige to slightly grey.
674	Mycelium thin, slightly woolly. Colony dark olive-grey. Reverse very dark olive-grey.
689	Mycelium dense, woolly. Colony light beige, with grey centre. Reverse beige, with greyish-orange centre.

Sporulation

All isolates sporulated abundantly on OMA, followed by CM. Sporulation on PDA and CMA was considerably variable. Isolates 630, 635, 645, 657, 660, 662, 674 and 689 sporulated abundantly on all media. Isolates 640, 659, 664, and 665 sporulated poorly on PDA and CMA. Isolates 634 and 659 failed to sporulate significantly on CMA (Table 6).

Table 6. Effect of growth medium on conidia production
of the 13 *Colletotrichum* isolates

	PDA	CMA	CM	OMA
630	+++	+++	+++	+++
634	++	-	++	+++
635	+++	+++	+++	+++
640	+	+	++	+++
645	+++	+++	+++	+++
657	+++	++	+++	+++
659	+	-	++	+++
660	+++	++	+++	+++
662	+++	++	+++	+++
664	+	+	++	+++
665	+	+	++	+++
674	+++	+++	+++	+++
689	+++	++	+++	+++

Key to size of pellet after centrifugation of conidial suspension:
- = none observed; + = very small; ++ = medium; +++ = large

Growth medium:
PDA - potato dextrose agar   CMA – corn meal agar
CM – Mathur's agar medium   OMA – oat meal agar

Colour of conidial mass

Differences in the colour of the conidial mass on OMA were also studied. OMA was used in this study because it produced the most conidia for all isolates. The colour of conidial mass can be divided into four groups, namely bright orange (630, 665), peach (634, 660, 664), salmon (635, 659), pale salmon (640, 645, 657, 662, 674, 689). However, it was difficult to differentiate between the colours due to very slight differences between them.

Setae production

The results of this study revealed that the formation and the abundance of setae in some isolates were dependent on the type of media used (Table 7). Isolates 657, 660, 662 and 674 produced abundant setae on all media, 640 produced setae on three media (CMA, CM and OMA), while 630 produced them on two media (PDA and CM). Isolate 635 produced setae only on OMA, and 645 only on CMA.

Number of nuclei in conidia

The results of DAPI staining are presented in Table 8. Almost all isolates (> 98 %) produced conidia which were predominantly uninucleate. Five of the isolates (634, 635, 657, 659 and 664) had a few conidia with two nuclei and of these, one (634), on occasional conidia, had 3 nuclei. The percentage of multinucleate

conidia was always very small. Even the isolate with large conidia (674) consistently had only one nucleus per conidium. Multinucleate conidia were observed to have variable shapes (usually longer than normal), and appeared to form by fragmentation of hyphal tips. None of the conidia was observed to contain more than 3 nuclei.

Table 7. Production of setae by the 13 *Colletotrichum* isolates on different media

Isolate	Media			
	PDA	CMA	CM	OMA
630	+	-	+	-
634	-	-	-	-
635	-	-	-	+
640	-	++	+	+++
645	-	+	-	-
657	+++	+++	++	+++
659	-	-	-	-
660	+++	+++	+++	+++
662	+++	+++	+++	+++
664	-	-	-	-
665	-	-	-	-
674	+++	+++	+++	+++
689	-	-	-	-

Key: - = setae absent; + = setae rare; ++ = setae moderate, +++ = setae abundant

Table 8. Percentage of conidia with one or more nuclei

Isolate	Percentage of conidia		
	1 nucleus	2 nuclei	3 nuclei
630	100.00		
634	98.84	1.15	0.01
635	99.95	0.05	
640	100.00		
645	100.00		
657	99.92	0.08	
659	99.98	0.02	
660	100.00		
662	100.00		
664	99.98	0.02	
665	100.00		
674	100.00		
689	100.00		

Discussion and conclusion

Environmental factors such as temperature, humidity and light play a major role in the development and spread of diseases caused by *C. gloeosporioides*. Studies have shown that high ambient temperatures (20–30 °C) and relative humidities

(85–100 %) prevalent in the tropics are the main factors contributing to disease outbreaks (Wastie 1972, Estrada 1990, Dodd *et al.* 1992). Another factor in the spread of disease is rainfall, which facilitates the dissemination of conidia. Fitzell and Peak (1984) studied the epidemiology of mango anthracnose in the field and concluded that the spread of the fungus within the mango canopies occurred by water-borne conidia. Work on the epidemiology of cacao anthracnose showed that although foliar infection could occur throughout the year, it increased sharply following the commencement of the rainy season (Chandra *et al.* 1989).

Most forest nurseries owned by state forest departments are small. Due to the limited space, seedlings in these nurseries are packed close together. This restricts air movement, which may enhance the humidity of the micro-environment. In addition, the seedlings are watered manually twice daily. This, coupled with the high ambient temperature (25–34 °C) and heavy rainfall prevalent at most times of the year create conducive environments for disease outbreaks and spread.

Accurate identification of a pathogen is crucial for the development of effective disease control measures. Correct identification is also important in the establishment of quarantine regulations, i.e. to ensure that the import, export and movement of a wide range of plant materials take place without the risk of dispersing dangerous plant pathogens (Bailey *et al.* 1995).

In the past, identification of *Colletotrichum* species was mostly based on morphological and cultural criteria, coupled with knowledge of the host origin of the pathogen. However, many isolates of *Colletotrichum* show extensive variation in culture (Sutton 1992, Bailey *et al.* 1995). At the same time, identification based on host range may also be unreliable as some species of *Colletotrichum* occur on a wide range of plant species (Shear & Wood 1913, von Arx 1957, Lenné 1978, Sutton 1980, 1992), while others are very host specific (Shear & Wood 1913, Tiffany & Gilman 1954, von Arx 1957, Simmonds 1965, Lenné 1978, Sutton 1980, 1992). As a result, many isolates with indistinguishable morphologies have been given different species names because of the host from which they were obtained (Sutton 1992).

Growth rate of cultures

Although limited use has been made of growth rate, several workers were able to utilise this criterion in distinguishing some species of *Colletotrichum*. For example, *C. musae* was noted for its comparatively rapid growth rate, while *C. lindemuthianum* was noted for its slow growth rate (Baxter *et al.* 1983). In their study, Smith and Black (1990) separated isolates of *C. acutatum* from *C. fragariae* and *C. gloeosporioides* by the slow growth rate of *C. acutatum* on PDA. Cox and Irwin (1988) found that the growth rates of isolates of *C. crassipes* were indistinguishable from those of *C. gloeosporioides*. As the rate of growth of most isolates in the present study were not significantly different (except for isolates 635 and 689), it was not possible to distinguish them using this criterion.

Although the rates of growth of most isolates in this study were not significantly different, two isolates, 635 and 689, were readily distinguished from the rest by

their slow growth. Morphological descriptions of isolate 635, obtained from this study, fit those of *C. gloeosporioides,* and interestingly, Edgerton (1908) and Lenné (1978) also reported working with a slow-growing *C. gloeosporioides,* but in both cases the isolates were obtained from apple.

Conidial morphology

Conidial morphology has always been emphasised over other taxonomic criteria in taxonomic investigations of the genus *Colletotrichum.* Workers such as Tiffany and Gilman (1954), Simmonds (1965), Mordue (1967, 1971), Sutton (1980, 1992) and Baxter *et al.* (1983) have all used conidial shape and size as the main criteria to distinguish a number of species. Although, based solely on conidial shape and size, von Arx (1957) managed to reduce drastically the number of species of *Colletotrichum* from several hundred to 11, including an exceptionally large number (almost 600) of synonyms for *C. gloeosporioides,* he was unable to distinguish species such as *C. lindemuthianum, C. musae, C. orbiculare* and *C. trifolii* from each other or from *C. gloeosporioides* (von Arx 1957, von Arx & van der Velden 1961).

The inadequacy of using conidial morphology alone is well-illustrated in the controversy surrounding the problem of *Colletotrichum* species on strawberries (Smith & Black 1990). These species, including *C. acutatum, C. gloeosporioides* and *C. fragariae,* are indistinguishable in terms of their conidial morphology. Although *C. acutatum* has been separated from *C. gloeosporioides* since its description by Simmonds (1965), confusion has arisen because of the extremely wide variation accepted for *C. gloeosporioides.* The situation was aggravated by the intermittent use of the name *C. fragariae,* an isolate poorly defined, but resembling *C. gloeosporioides* (Howard & Albregts 1983).

The present study illustrated variation in conidial size in all the isolates and the overlapping ranges of size of many. This agrees with Baxter *et al.* (1983), who indicated that the range of conidial size tends to overlap between different species and suggested that a mean value was more acceptable. The variability of conidial size within isolates could be increased through the presence of "giant conidia", the term given by Cox and Irwin (1988), to described conidia produced by hyphae. Variability in conidial size was observed by Davis *et al.* (1992) within isolates of *C. gloeosporioides* from *Stylosanthes.* Sutton (1980) indicated the difficulties associated with providing a standardised description for such a variable species as *C. gloeosporioides* and considered it as a group species. This is because elements within it could not be separated satisfactorily using criteria such as conidial shape and size. Several other workers, including Gorter (1962), Simmonds (1965), Hindorf (1970), Mordue (1971) and Ogle *et al.* (1986), have also noted the heterogeneity of *C. gloeosporioides.*

With the exception of one isolate (674) with very big conidia, the shape and mean size range (12.7–22.2 µm × 4.6–5.5µm) of most isolates from the forest trees seedlings were in the range of *C. gloeosporioides* described by von Arx (1957) and Mordue (1971). Those of isolate 689, which had conidia with acute apices

and narrow width, fell within the morphological descriptions and range of *C. acutatum* (Sutton 1980, Baxter *et al.* 1983, Smith & Black 1990, Gunnel & Gubler 1992, Sutton 1992).

Germination and appressorial formation

The slight swelling of conidia prior to germination was consistent with reports by Lenné (1978). However, Dey (1933) did not observed this occurrence in his study on *C. gloeosporioides* and Hawker (1966) found that swelling was not common in spores which germinated in water. It is a generally held view that conidia of all species of *Colletotrichum* become septate following mitosis, just prior to germination (Dey 1933, Skoropad 1967, Lenné 1978, Sutton 1980, Baxter *et al.* 1983, Jeffries *et al.* 1990). However, for species such as *C. lindemuthianum, C. orbiculare* and *C. trifolii*, conidia remain aseptate during germination (O'Connell *et al.* 1992). Lenné (1978) also reported the lack of septation in an isolate of *C. lindemuthianum*. This unique morphological feature has been used to distinguish this group of species from all the other species of *Colletotrichum* (Sherriff *et al.* 1994, Bailey *et al.* 1995). Since all the isolates in this study were septate, this suggests that none of them were *C. lindemuthianum, C. malvarum, C. orbiculare* or *C. trifolii*.

The occurrence of microcyclic conidiation, the process whereby sporulation occurs directly after conidia germination with a greatly reduced vegetative phase, was observed in a few isolates in this study, similar to those observed by Slade *et al.* (1987). Considerable research has been done on microcyclic conidiation and much of these researches have indicated that this phenomenon is induced by high inoculum density, optimum temperature and favourable nutrient-limited media. This rapid and highly efficient sporulation capability is very desirable for the commercial mass production of fungal conidia required for biological control agents (Churchill 1982).

Appressorial morphology

Appressorial morphology is regarded by many workers as an important morphological criterion in distinguishing species of *Colletotrichum* (von Arx 1957, Sutton 1962, 1980, Baxter *et al.* 1983, Cox & Irwin 1988). Lenné (1978) found that the size, shape and colour of appressoria varied among species groups. In the present study, only appressoria produced by conidia were examined. This is because the shape of those produced by hyphae was too complex to be used as taxonomic criteria (Maziah 1990). This is consistent with Lenné's (1978) observation that hyphal appressoria were variable and of no taxonomic value. In contrast, Sutton (1962, 1968) has used hyphal appressoria very extensively, especially to distinguish *C. dematium* from *C. trichellum* and in separating the *C. graminicola* groups.

In this work, the shapes of conidial appressoria were categorised into four types: entirely globose, globose to subglobose, subglobose to slightly lobed and obviously

lobed. Apart from the two extremes (entirely globose and obviously lobed), appressoria shape of the intermediate types could not separate the isolates. Lenné (1978) observed two types of appressoria within *C. gloeosporioides*: the ellipsoid to ovoid type and the subglobose to ellipsoid type, while Sutton (1992) described appressoria of *C. gloeosporioides* as clavate, ovate, obovate and sometimes lobed. Gunnel and Gubler (1992) found that the appressoria of *C. fragariae*, *C. gloeosporioides* and *C. acutatum* produced in culture were not useful in defining species as they were similar in size and varied from smooth to lobed within a species. Simmonds (1965) and Baxter *et al.* (1983) found the appressorial shapes and dimensions of *Colletotrichum* species to be variable, precluding their use as taxonomic criteria.

By combining the use of conidial dimension and appressorial morphology, Cox and Irwin (1988) suggested the existence of three biological groups within isolates which were originally classified as belonging to *C. gloeosporioides:* (1) those isolates with mean conidial widths between 3 and 4.2 μm and with either unlobed or slightly lobed appressoria; (2) isolates with mean conidial widths between 4.5 and 5.5 μm and with unlobed or slightly lobed appressoria; and (3) isolates with conidial widths between 4.5 and 5.5 μm but with obviously lobed appressoria. The last group is compatible with current definitions of *C. crassipes* (Sutton 1980). In the present study, there was evidence that isolates with a high percentage of obviously lobed appressoria (660, 662 and 674) were compatible with the above definition of *C. crassipes*. The observation that large and obviously lobed appressoria were mostly deeply melanised was in agreement with Lenné (1978) and Ogle *et al.* (1986).

Setal and perithecial morphology

Shear and Wood (1913) rejected setal production as a taxonomic criterion because of its variability in one species, *C. gloeosporioides*. On the same basis, von Arx (1957) rejected the presence of setae as a distinction between the form genera *Gloeosporium sensu Saccardo* and *Colletotrichum* which were recognised as synonyms. However, in 1962, Sutton suggested that setae production was a basic taxonomic requirement for species within the genus *Colletotrichum*. Lenné (1978) confirmed the conclusions of Shear and Wood (1913), but in regard to *C. gloeosporioides* only. With the exception of *C. orbiculare*, which was highly variable, all other species she studied were consistent in producing setae or lacking setae.

In the present study, setal morphology was found to be very variable within the 13 isolates examined. This is in agreement with previous workers who have also observed the variability of setal morphology in *C. gloeosporioides* (Shear & Wood 1913, Lenné 1978). Setal availability and abundance was found to be dependent upon the type of medium used. Similar observations have also been reported by Kruger (1913) (cited in Lenné 1978). Analysis of setae dimensions indicates that two groups were distinct. One group had setal lengths ranging from 52.80 to 96.00 μm and the other ranging from 116.80 to 130.30 μm. Gunnel and Gubler (1992) demonstrated that setal morphology is an important criterion

in distinguishing three closely relates species, i.e. *C. fragariae, C. acutatum* and *C. gloeosporioides.* In contrast, Baxter *et al.* (1983) observed that setal morphology was similar in all species they examined and did not prove to be discriminatory.

Production of conidia by setae observed with a few isolates in these studies was also reported to occur in several *Colletotrichum* species (Lenné 1978, Lenné *et al.* 1984, Maziah 1990, Smith & Black 1990, Gunnel & Gubler 1992). The first observation of conidial production by setae was recorded on *C. gossypii* by Southworth (1891), who also noted that conidia produced from setae were somewhat smaller than those borne on conidiophores. Fertile setae were readily distinguished by their truncate, near-hyaline apices from the darker, usually pointed apices of sterile setae. Lenné (1978) speculated that setae might be a survival mechanism which allowed the fungus to survive cold and dry conditions, or it might be a conidial dispersal mechanism during humid, windy conditions (Lenné 1978).

An extensive synonymy exists (over 120) for *Glomerella cingulata.* Sexuality in *G. cingulata* is complex, with homothallic and heterothallic forms being recorded. In the present study only the presence and absence of the sexual stage were taken into consideration and although a number of isolates produced perithecia, very few produced fertile perithecia. Among those that produced fertile perithecia, all seemed homothallic. The perithecia and ascospore morphology were indistinguishable. This is in agreement with von Arx and Muller (1954) who showed that the morphology of many *Glomerella* species was similar and indistinguishable from that of the type species *G. cingulata.*

In this study, the presence of sterile perithecia was common in some isolates. These perithecia were morphologically indistinguishable from the fertile form. This observation is consistent with studies by Irwin and Cameron (1978) who observed the common occurrence of sterile perithecia in pure cultures of Type A isolates of *C. gloeosporioides* from *Stylosanthes.* Gunnel and Gubler (1992) observed sterile perithecia in the isolates which had been previously identified as *C. fragariae.* However, the production of fertile perithecia by these isolates indicates that they were *C. gloeosporioides.*

Cultural characteristics

Cultural characters have been used by several early workers as taxonomic criteria to compare species of *Colletotrichum* (Edgerton 1908, Shear & Wood 1913). Later, workers such as Tiffany and Gilman (1954), von Arx (1957), Lenné (1978) and Sutton (1980) placed considerable importance on cultural characters as a taxonomic criterion. Baxter *et al.* (1983) also regarded cultural characteristics as a good taxonomic criterion in distinguishing species of *Colletotrichum.* Nevertheless, Bailey *et al.* (1995) argued that many isolates of *Colletotrichum* often show extensive variation in culture and furthermore, the culture conditions, including the media, the age of culture and the temperatures used, cannot be standard between laboratories (Sutton 1992).

The colour of conidia of most isolates in this study ranged from pale salmon to peach. Because the range of colours among isolates overlapped and they were indistinguishable, colour of conidial mass could not be used as a means of separation.

In this study, CM was found to be the most suitable medium for growth and sporulation of all the isolates as they were less variable on this medium than on others. However it is not ideal for the study of culture characteristics because of the lack of mycelial growth. In all isolates, conidial production was most abundant on OMA. This agrees with the study by Lenné (1978) who also found that OMA was superior to PDA and MEA for comparing and distinguishing *Colletotrichum* in culture. She also noted that *C. gloeosporioides* was less variable on OMA than on other media.

Generally, results from the present culture characteristic studies suggest that these criteria are of little value in distinguishing the isolates obtained from forest trees. This agrees with some early researchers, whose studies were generally restricted to *C. gloeosporioides* and who noted the great variability within this species growing in culture (Stoneman 1898, Edgerton 1908, Shear & Wood 1913). Other criteria such as the presence and dimensions of setae and colour of conidial mass were also too variable or too similar to make them useful taxonomic criteria.

Nuclear composition

The number of nuclei in conidia and mycelium of several species of *Colletotrichum* has been reported (Politis & Wheeler 1973, Suzuki *et al.* 1982, Russo & Pappelis 1984). True phialo-conidia of *C. atramentarium* (=*C. coccodes*) (Griffiths & Campbell 1972, 1973), and *C. truncatum* (Staples *et al.* 1976) were observed to be uninucleate when grown on solid media. However, the number of nuclei in spores of several species of *Colletotrichum* was reported to be variable when produced in liquid media, due to the production of blastospores and binary fission spores apart from having the true phialo-conidia (Churchill 1982).

In the present study, conidia produced on agar media were predominantly uninucleate (>98 %). This result is consistent with those of previous workers (Akai & Ishida 1967, Griffiths & Campbell 1972, 1973, Politis & Wheeler 1973, Staples *et al.* 1976, TeBeest *et al.* 1989). However, a very small percentage of multinucleate conidia was also produced by some isolates. These conidia were larger than normal and resembled the spores described by Churchill (1982). According to TeBeest *et al.* (1989), the multinucleate conidia-like cells were results of division of nucleus without division of the conidia or from pinching-off of hyphal tips as was observed in this study. In another study, Churchill (1982) estimated that only 8–10 % of the conidia of *C. gloeosporioides* f. sp. *aeschynomene* produced in liquid cultures were true conidia, while 80–85 % of the other were derived from fragmentation.

It is concluded that the nuclear number in conidia is of no significance in distinguishing isolates of *Colletotrichum* species. However, it is an important

consideration when protoplast fusion or plasmid mediated recombination experiments are conducted since it is necessary to isolate uninucleate cells (TeBeest *et al.* 1989)

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