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Comparative Studies of Isolates of *Colletotrichum gloeosporioides* from Eighteen Malaysian Hosts

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ABSTRAK

Colletotrichum gloeosporioides *daripada lapan belas perumah di Malaysia yang terdiri daripada oren* (Citrus reticulata)*, koko* (Theobroma cacao)*, orkid* (Cattleya *sp.*)*, rumput* (Imperata cylindrica)*, lada hitam* (Piper nigrum)*, cili* (Capsicum annuμm)*, mango* (Mangifera indica) *dan kekacang* (legum) *iaitu tanaman penutup bumi* (Pueraria phaeseoloides, Centrosema pubescens *dan* Calopogonium mucunoides) *dan rumpair* (Mimosa pudica)*, sayur-sayuran* (Psophocarpus tetragonolobus, Phaseolus vulgaris, Vigna radiata, Vigna sesquipedalis *dan* Arachis hypogaea)*, pokok renik* (Leucaena leucocephala) *dan pokok herba* (Clitoria ternatea)*, telah dikaji dari segi ciri-ciri pertumbuhan koloni, morfologi konidia, pertumbuhan pada berbagai media, suhu dan pathogenisiti ke atas hipokotil kacang* Phaseolus. *Saiz konidium* Colletotrichum gloeosporioides *berada di antara 14.25-19.0 × 2.7-5.03 μm. Saiz-saiz appressorium di antara 6.34-10.08 × 5.28-7.31 μm dan ianya berbentuk globus/subglobus/lobus. Tiada korelasi di antara saiz dan bentuk appressorium. Suhu optimum untuk pertumbuhan adalah 28 dan 30°C. Tiada satu pun diantara isolat-isolat tersebut yang menyebabkan simptom-simptom infeksi pada hipokotil kacang* Phaseolus.

ABSTRACT

Colletotrichum gloeosporioides *from eighteen Malaysian hosts, namely mandarin orange* (Citrus reticulata)*, cacao* (Theobroma cacao)*, an orchid* (Cattleya *sp.*)*, pepper* (Piper nigrum)*, chilli* (Capsicum annum)*, grass* (Imperata cylindrica)*, mango* (Mangifera indica) *and legume cover crops* (Pueraria phaeseoloides, Centrosema pubescens, *and* Calopogonium mucunoides) *and a weed* (Mimosa pudica)*, vegetables* (Psophocarpus tetragonolobus, Phaseolus vulgaris, Vigna radiata, Vigna sesquipedalis *and* Arachis hypogaea)*, a shrub* (Leucaena leucocephala) *and a herbaceous vine* (Clitoria ternatea) *were examined for colony growth characteristics, morphology of conidia, growth on various media and temperatures and pathogenicity on* Phaseolus *bean hypocotyls. Conidium size of* Colletotrichum gloeosporioides *was 14.25-19.0 × 2.7-5.03 μm. The appressorium size was 6.34-10.08 × 5.28-7.31 μm and the shape was globose/sub-globose/lobed. No correlation between the appressorium size and shape was noticed. The optimum temperature for growth was 28 and 30°C. None of the isolates caused infection symptoms on* Phaseolus *bean hypocotyls.*

INTRODUCTION

Colletotrichum gloeosporioides (Penz.) Penz. & Saccs. causes anthracnose disease of flowers, fruits and leaves of various plants, causing serious postharvest damage of many tropical fruits such as mango, citrus, avocado, papaya, crops like cacao (Mordue 1971; Sutton 1980) and legumes such as *Stylosanthes* (Irwin and Cameroon 1978; Davis *et al.* 1992). The taxonomy of *C. gloeosporioides*, according to Sutton (1980), is based mainly on conidial morphology, which is extremely variable. Many authors have proposed groupings of *C. gloeosporioides* from tropical fruit crops (Hodson *et al.* 1993) and of *Stylosanthes* spp. in Australia (Dale *et al.* 1988; Braithwaite *et al.* 1990; Davis *et al.* 1992). These workers have used

conidial morphology, colony characters, disease symptoms, double stranded RNA and ribosomal and mitochondrial DNA polymorphisms to characterize the variablity among *C. gloeosporioides* isolates.

In Malaysia, tropical fruits are many and varied, while many legumes exist wild as well as cultivated and are used as vegetables, cover crops and as ornamental plants. *C. gloeosporioides* is present on the stems, leaves, flowers and fruits of many plants.

This present study deals with the *in vitro* examination of *C. gloeosporioides* to assess the extent of morphological and cultural variation within isolates of 18 Malaysian hosts.

MATERIALS AND METHODS

Isolates of *Colletotrichum gloeosporioides* were obtained from mandarin orange (*Citrus reticulata*), cacao (*Theobroma cacao*), an orchid (*Cattleya* sp.), pepper (*Piper nigrum*), chilli (Capsicum annuum), lallang grass (*Imperata cylindrica*), mango (*Mangifera indica*) and legumes: cover crops (*Pueraria phaeseoloides, Centrosema pubescens* and *Calopogonium mucunoides*), the weed *Mimosa pudica*, vegetables (*Psophocarpus tetragonolobus, Phaseolus vulgaris, Vigna radiata, Vigna sesquipedalis, Arachis hypogaea*), the shrub (*Leucaena leucocephala*) and the herbaceous vine (*Clitoria ternatea*). As almost all isolates of individual hosts had a similar appearance, only one isolate of each host was chosen for this study. Single spore colonies of each isolate were maintained on potato dextrose agar (PDA OXOID) until required.

Colony Characteristics and Growth Rates

The 18 isolates were grown on potato dextrose agar (PDA) at 28°C. A disc, 5 mm in diameter, of the fungal mycelium of each isolate was taken from the growing edge of 5-day-old colonies and transferred to the centre of PDA in 9-cm plastic petri dishes. Two replicate plates were made for each isolate. The plates were incubated for 5 days at 28°C. The diameter of the resulting colony was measured each day and the growth rate for each isolate was calculated. The colony characteristics were described using colony features such as mycelium, reproductive structures and colony appearances (modified from Davies *et al.* 1992).

Conidial and Appressorial Morphology

The isolates were grown on glucose caesamino acid medium at 28°C for 5 days under light. A spore suspension was prepared by the addition of 10 ml of sterile distilled water and agitating the colony surface. The spore suspension was filtered through muslin cloth, centrifuged at 1000 g for 4 min twice; the resultant pellet was resuspended in sterile distilled water to obtain a final concentration of 1×10^4 conidia per ml of water. The suspension was then (a) examined microscopically and the length and the width of at least 50 conidia per isolate were measured, (b) drops of conidial suspension, 10 µl in volume for each isolate, were placed on welled slides and incubated under moist conditions at 25°C for 12 h. The shape and size of the appressoria produced by the germinated conidia were recorded, and (c) slides from (b) were then flooded with 0.02% (w/v) aq. Calcofluor White M2R for 3 min, rinsed once with water and then examined under epi-fluorescence microscopy. The presence or absence of septa on the germinated conidia was noted.

Growth Studies

The effects of media and temperature on the growth of the colony of the different isolates were tested.

Media Effect on Colony Growth

The solid media used in the experiment

were potato dextrose agar (PDA), malt agar (MA), lima bean agar (LBA), oatmeal agar (OMA) and Czapek Dox agar (CDA-Difco). Two replicate plates of each colony for each isolate were prepared. A mycelial plug, 5 mm in diameter, was cut from a 7-day-old colony of each isolate and plated on freshly prepared agar plates of each of the media. Two radial diameters of the colonies were measured at right angles to one another after incubation at 28°C for 5 days.

Temperature Effect on Colony Growth

The temperatures studied were 15, 20, 25, 28, 30 and 35°C. Mycelial plugs 5 mm in diameter were transferred to freshly prepared PDA plates and the plates incubated at the above temperatures. The colony diameters of growth at two right angles were measured after 5 days. Two replicate plates were used for each isolate, and the experiment was repeated three times. Graphs were drawn using the average measurement of growth at each temperature, and the optimum temperature required for growth of each of the fungal isolates was determined from the graphs.

Pathogenicity Tests

The spore suspension 1×10^4 of each of the isolates was prepared after washing 3 times in deionised distilled water. Drops of 7 µl of these spore suspensions were used as inoculum on 5 points of a bean hypocotyl cut from 7-day-old seedlings of *Phaseolus vulgaris*. The ends of the hypocotyls were sealed in molten wax to prevent drying. Five hypocotyls were used for each isolate. Another 5 hypocotyls were inoculated with sterile deionised water and were used as controls. Prior to inoculation all hypocotyls were arranged on a rack and placed in plastic containers lined with moist tissue paper. The boxes were incubated under moist conditions at 25°C and the hypocotyls were examined periodically on Day 5, 7 and 14 after inoculation. The hypocotyls were first examined visually, then under a low-powered microscope to estimate the extent of lesion formation on the hypocotyl. An epidermal peel of the inoculated regions was also examined under a high-powered microscope and photomicrographs were taken.

RESULTS AND DISCUSSION

Colony characters, especially the appearance, colour and mycelial form, of the isolates of *C. gloeosporioides* from the 18 isolates varied greatly. The colour of *C. gloeosporioides* varied from white, to grey, to dark orange or pink-grey, while the reverse side of the colonies was of white, dark grey, orange or a mixture. Most colony margins were regular. Almost all isolates grew well on PDA with a growth rate of 11.00 – 15.91 mm per day except an isolate from *Cattleya* which had a growth rate of 7.00 mm per day (*Fig 1*). The mycelium was hyaline,

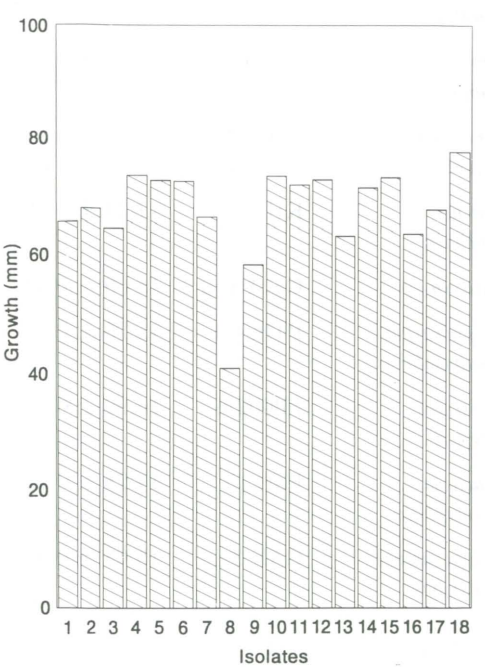

Fig. 1 Growth of C. gloeosporioides *on PDA at 28°C after 5 days*

TABLE 1
Size (μm) of conidia and appressoria of *Colletotrichum gloeosporioides* isolates

Isolates		Conidia		Appressorium		
Hosts	Code/ number	Length	Width	Length	Width	Shape**
Imperata cylindrica	Imper C/1	17.38 ± 0.44	4.99 ± 0.13	8.33 ± 0.43	7.18 ± 0.32	G
Citrus reticulata	CM/9	16.19 ± 0.24	4.09 ± 0.15	7.19 ± 0.26	5.40 ± 0.18	G/SG
Mimosa pudica	MPFI/2	17.10 ± 0.49	4.49 ± 0.13	9.19 ± 0.27	6.38 ± 0.27	G/SG
Vigna sesquipedalis	VSL2/18	17.35 ± 0.61	5.03 ± 0.11	7.80 ± 0.89	5.85 ± 0.17	G/SG
Pueraria phaseoloides	Peu B/10	15.69 ± 0.37	4.15 ± 0.25	9.08 ± 0.18	5.84 ± 0.15	G/SG
Piper nigrum	PipB003/11	14.31 ± 0.29	4.33 ± 0.13	6.55 ± 0.27	5.28 ± 0.17	SG
Vigna radiata	Khst/5	19.00 ± 1.19	4.31 ± 0.24	6.34 ± 0.25	5.33 ± 0.17	L
Theobroma cacao	CPO12/16	17.28 ± 1.23	4.68 ± 0.28	7.38 ± 0.15	5.38 ± 0.17	SG
Mangifera indica	M003/12	15.56 ± 0.44	4.48 ± 0.12	7.46 ± 0.27	6.34 ± 0.25	G
Clitoria ternatea	CIB010/13	14.93 ± 0.26	4.58 ± 0.11	10.08 ± 0.30	7.31 ± 0.11	SG
Capsicum annuum	Chi010/17	18.85 ± 0.35	2.70 ± 0.56	7.50 ± 0.20	6.20 ± 0.23	SG/L
Centrosema pubescens	Censt/14	16.81 ± 0.31	4.95 ± 0.05	9.90 ± 0.32	5.63 ± 0.14	SG/L
Arachis hypogaea	KtL/7	17.34 ± 0.15	4.54 ± 0.12	9.19 ± 0.20	6.56 ± 0.17	SG
Cattleya sp.	Cat CL/8	15.69 ± 0.52	4.00 ± 0.09	7.25 ± 0.14	6.15 ± 0.13	G/SG
Psophocarpus tetragonolobus	PT004/4	15.26 ± 0.27	4.59 ± 0.13	6.58 ± 0.08	5.85 ± 0.11	G/SG/L
Phaseolus vulgaris	FB002/6	16.99 ± 0.45	4.54 ± 0.09	6.70 ± 0.25	5.42 ± 0.11	G/SG/L
Leucaena leucocephala	PbL/15	14.25 ± 0.52	4.76 ± 0.10	8.24 ± 0.23	6.38 ± 0.24	SG/G
Calopogonium mucunoides	CAF/3	15.63 ± 0.94	4.88 ± 0.18	7.38 ± 0.33	5.50 ± 0.19	SG/G

Comfidence limit = 95%
* SEM
** G = Globose SG = Sub-globose L = Lobed

brown or both, sometimes abundant, at times sparse with either floccose, loose or compact growth. Some colonies formed sclerotia. Conidia formation was either on the hypha or in the acervulus, either only centrally or radially throughout the colony. The acervuli were either hyaline or dark, and at times a few dark setae were seen on the acervuli. Perithecia were formed by isolates from *Phaseolus vulgaris* and *Psophocarpus tetragonolobus*. The conidia were cylindric with obtuse ends or ovoid, the size varying from 14.25 – 19.0 μm × 2.7 – 5.03 μm (Table 1). The longest (19.0 μm) was produced by the *V. radiata* isolate and the shortest conidium (14.30 μm) by the isolates of *Piper nigrum* and *L. leucocephala*. All isolates produced conidia 4.0 – 5.03 μm in width, except for that of *C. annuum* with a width as low as 2.7 μm (Table 1). The spore measurements of the ovoid conidia of the above isolates fit within the measurements of spore sizes of *C. gloeosporioides* as given by Sutton (1980) and Mordue (1971). Giant conidia reported by Davis *et al.* (1992) were not found in this study of Malaysian isolates.

Germinated conidia of all isolates of *C. gloeosporioides* showed the presence of a septum which was very clearly seen after treatment with the fluorescent brightener, calcofluor (*Plate 1*). This character is said to be shown by all ovoid condia of *Colletotrichum* species except for *C. lindemuthianum* (O'Connell *et al.* 1992). Hence the isolates studied are definitely not *C. lindemuthianum* although the spores were cylidrical in shape and the majority were isolated from legumes.

The shape of the appressoria produced by the germinated conidia was variable, from globose (G), sub-globose (SG) to lobed (L). Size of the appressoria ranged from 6.34 – 10.08 μm long to 5.28 – 7.31 μm wide. Short, small appressoria (6.35 – 6.70 μm by 5.28 – 5.85 μm were produced by the isolates of *Phaseolus vulgaris*, *Psophocarpus tetragonolobus*, *V. radiata* and *Piper*

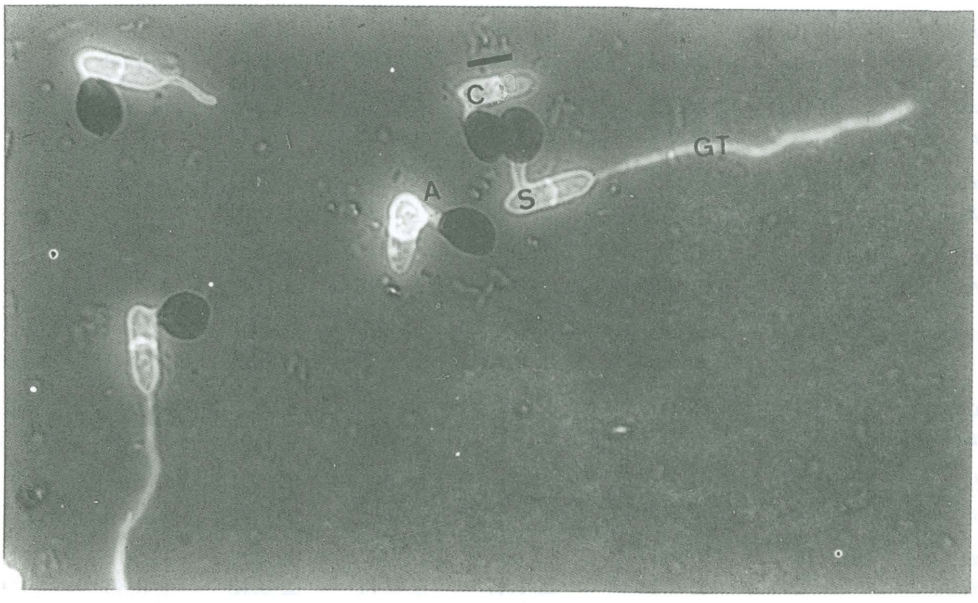

Plate 1. Germinating conidia of Colletotrichum gloeosporioides *on glass slides stained with 0.02% (w/v) aq. Calcofluor White M2R and viewed with epifluorescence microscopy*

A = appressorium, C = conidium, GT = germ tube, S = septum. Scale: 1cm = 10 μm

TABLE 2
Diameter of radial growth of *Colletotrichum gloeosporioides* isolates after five days

Isolates		Mean Diameter of Colony (mm)					
Hosts	Code/ number	MA	LBA	MEA	CDA	OMA	PDA
Imperata cylindrica	ImperC/1	61.00 ± 0.19*	53.25 ± 0.63	62.25 ± 0.63	66.50 ± 0.96	70.00 ± 1.41	66.25 ± 1.89
Citrus reticulata	CM/9	67.50 ± 2.06	69.25 ± 1.13	63.75 ± 0.85	59.75 ± 0.75	67.00 ± 1.58	58.88 ± 1.71
Mimosa pudica	MPF1/2	72.25 ± 1.32	71.00 ± 0.41	73.25 ± 0.75	65.25 ± 0.63	69.75 ± 2.87	68.50 ± 0.65
Vigna sesquipedalis	VSL2/18	80.75 ± 0.48	77.50 ± 1.19	69.50 ± 0.29	81.25 ± 2.66	81.50 ± 2.53	78.13 ± 0.32
Pueraria phaseoloides	Peu B/10	75.00 ± 2.04	81.75 ± 0.25	74.25 ± 0.25	70.25 ± 0.75	74.50 ± 0.29	74.75 ± 2.15
Piper nigrum	PipiB003/11	71.25 ± 1.03	81.50 ± 2.53	74.00 ± 0.91	69.25 ± 1.03	73.00 ± 1.16	72.50 ± 1.31
Vigna radiata	Khst/5	74.25 ± 1.12	71.50 ± 0.29	74.75 ± 0.48	74.00 ± 0.58	69.50 ± 3.52	73.25 ± 0.88
Theobroma cacao	CPO12/16	69.50 ± 0.65	68.50 ± 0.29	64.50 ± 1.76	64.75 ± 1.93	73.50 ± 3.58	64.13 ± 0.89
Mangifera indica	M003/12	68.25 ± 3.04	74.75 ± 0.48	71.75 ± 0.85	69.50 ± 0.29	54.25 ± 1.03	73.38 ± 1.69
Clitoria ternatea	CLB010/13	64.00 ± 1.47	73.50 ± 0.29	72.75 ± 0.85	61.50 ± 0.96	69.25 ± 1.44	63.75 ± 0.52
Capsicum annuum	Chi010/17	78.75 ± 0.25	76.50 ± 1.50	79.25 ± 0.63	71.25 ± 1.11	74.50 ± 0.65	68.50 ± 1.30
Centrosema pubescens	Censt/14	71.25 ± 2.50	67.00 ± 0.41	68.25 ± 0.75	65.25 ± 0.85	70.25 ± 0.25	72.00 ± 1.14
Arachis hypogaea	KtL/7	63.75 ± 0.25	74.00 ± 0.41	70.75 ± 0.48	78.00 ± 0.41	62.25 ± 4.92	67.00 ± 1.62
Cattleya sp.	Cat CL/8	52.50 ± 1.04	53.25 ± 2.18	49.50 ± 0.29	69.50 ± 0.29	53.00 ± 0.41	41.13 ± 0.55
Psophocarpus tetragonolobus	PT004/4	74.00 ± 0.36	71.63 ± 0.24	58.98 ± 0.88	62.50 ± 0.65	65.00 ± 0.98	74.13 ± 1.20
Phaseolus vulgaris	FB002/6	74.75 ± 0.86	71.75 ± 0.48	58.00 ± 0.41	62.50 ± 0.29	65.25 ± 0.48	73.13 ± 0.88
Leucaena leucocephala	PBL/15	79.00 ± 0.41	77.00 ± 1.47	74.00 ± 0.41	69.25 ± 0.48	73.50 ± 0.65	73.00 ± 0.41
Calopogonium mucunoides	CAF/3	69.00 ± 0.41	71.25 ± 1.18	65.75 ± 0.25	60.25 ± 0.48	70.00 ± 0.00	65.13 ± 1.25

Confidence limits = 95% *SEM

nigrum, while the largest were produced by the isolate from *Clitoria* (Table 1). There was no correlation between the size and shape of the appressoria although this had been reported by Cox and Irwin (1988).

All isolates grew well on all media tested although there were a few significant differences (Table 2). The growth of the isolate from *Cattleya* was relatively lower on most media except CDA compared with the growth of all other isolates. All isolates from legumes showed very good growth after 5 days (> 60 mm), some attaining > 80 mm as shown by the isolate from *V. sesquipedalis* on MA, CDA, OMA, and from *Pueraria* on LBA. The isolate from *Piper nigrum* also reached over 80 mm diameter in 5 days. All media supported good growth of *C. gloeosporioides*, with the growth rate on PDA at 28°C of all isolates varying slightly between 11.00 to 15.91 mm per day except for the slow growing isolate from *Cattleya* with a growth rate of 7.00 mm per day (Table 2).

Two optimum temperatures for growth were obtained for the isolates under study: (i) the optimum temperature for the 9 isolates from *Capsicum annuum, Vigna radiata, V. sesquipedalis, Pueraria phaseoloides, Calopogonium muconoides, Centrosema pubescens, Psophocarpus tetragonolobus, Clitoria ternatea* and *Leucaena leucocephala* was 28°C for growth on PDA, (ii) for the isolates from *Cattleya, Citrus reticulata, Imperata cylindrica, T. cacao, Piper nigrum, Mangifera indica, Phaseolus vulgaris, Mimosa pudica* and *Arachis hypogaea* the optimum temperature was 30°C (*Fig. 2a, b*). In general, *C. gloeosporioides* grows well between temperatures of 25-30°C but at 15 and 35°C growth was reduced. The optimum temperatures for growth of *C. gloeosporioides* on Malaysian hosts agree

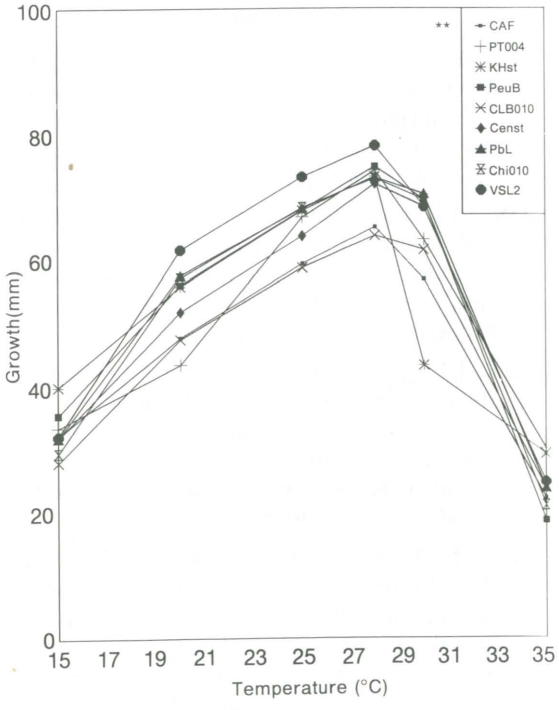

Fig. 2a. Temperature effect on the radial growth of Colletotrichum gloeosporioides *on PDA after 5 days*
**Isolates that showed optimum growth at 28°C

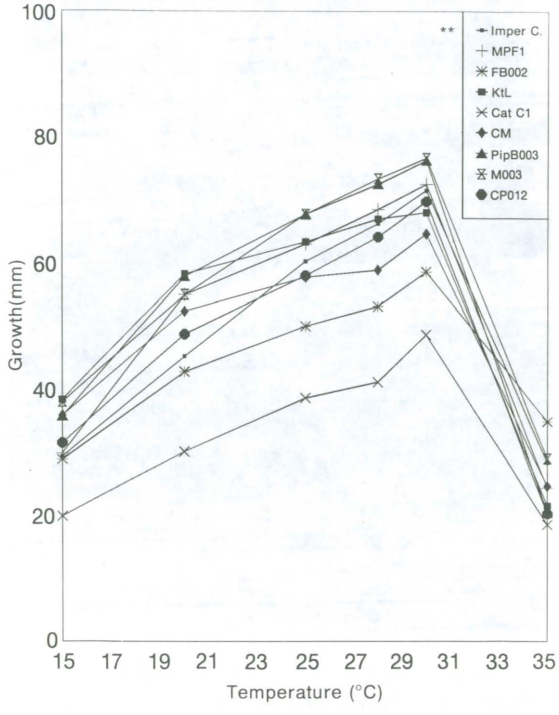

Fig. 2b. Temperature effect on the radial growth of Colletotrichum gloeosporioides *on PDA after 5 days*
**Isolates that showed optimum growth at 30°C.

with the results of previous workers (Cox and Irwin 1988; Davis *et al.* 1992).

All the bean hypocotyls inoculated with the various isolates of *C. gloeosporioides* showed slight brown discoloration as tiny brown spots over the epidermal cells of the inoculated regions. The intensity of the epidermal spots varied only slightly between the isolates. Examination of the epidermis showed germinated conidia and germ tubes ending with appressoria. Water-soaked regions appeared 5-7 days after inoculation and by Day 14 acervuli with pink glistening spore masses appeared over the entire hypocotyl length. Some acervuli had black setae. When viewed under the microscope the epidermal cells appeared to contain brown inclusions (*Plate 2*), indicating host reaction to infection. Although brown spots or dicolorations were seen, no further disease symptoms were observed. The subsequent water-soaked regions may be a consequence of weakened condition of the hypocotyls due to the incision on the hypocotyls or of ageing, which allows the mycelium to penetrate and colonize the soft, weakened plant tissues before forming the acervuli and conidia.

Plate 2. Surface view of the epidermal layer of Phaseolus vulgaris *hypocotyl 7 days after inoculation with conidia of* Colletotrichum gloeosporioides

A = appressorium, E = epidermal cell, EB = browned epidermal cell. Scale: 1cm = 10 μm

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